IL-1β and TNF-α induce MUC5AC overexpression through a mechanism involving a sequential activation of ERK/p38 MAP kinases-MSK1-CREB in human airway epithelial cells

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLE

LIST OF ABBREVIATIONS

MUC: mucin

MAP kinase: mitogen-activated protein kinase

ERK: extracellular signal-regulated kinase

JNK: c-Jun N-terminal kinase

MEK1: MAPK/ERK kinase 1

SAPK: Stress-activated protein kinase

MAPKAP-K: MAP kinase-activated protein-kinase

MSK1: mitogen-and stress-activated protein kinase 1

CRE: cAMP response element

CREB: CRE-binding protein

DN: dominant-negative

IL: Interleukin

TNF: tumor necrosis factor

ATF2: activating transcription factor 2

IBMX: 3-isobutyl-1-methylxanthine

ABSTRACT

IL-1β and TNF-α induce MUC5AC overexpression through a mechanism involving a sequential activation of ERK/p38 MAP kinases-MSK1-CREB in human airway epithelial cells

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(Directed by professor Joo-Heon Yoon)

Mucin hypersecretion is commonly observed in many inflammatory diseases of the respiratory tract. *MUC5AC* is generally recognized to be a major airway mucin because *MUC5AC* is highly expressed in the goblet cells of human airway epithelium. Moreover, it is regulated by various inflammatory cytokines.

However, the mechanisms by which the interleukin (IL)-1β and tumor necrosis factor (TNF)-α induce *MUC5AC* gene expression in normal nasal epithelial cells, and the signal molecules involved, especially in the downstream signaling of mitogen-activated protein (MAP) kinases, remain unclear. Here we show that pharmacologic or genetic inhibition of either ERK or p38 MAP kinase pathway abolished IL-1β- and TNF-α-induced *MUC5AC* gene expression in normal human nasal epithelial cells. Our results also indicate that the activation of mitogen- and stress-activated protein kinase 1 (MSK1) and cAMP response element-binding protein (CREB) and CRE signaling cascades via ERK and p38 MAP kinases are crucial aspects of the intracellular mechanisms that mediate *MUC5AC* gene expression. Taken together, these studies give additional insights into the molecular mechanism of IL-1β- and TNF-α-induced *MUC5AC* gene expression and will enhance our understanding on mucin hypersecretion during inflammation.

Key words: CRE, CREB, ERK, MSK1, *MUC5AC*, p38.

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I. INTRODUCTION

Mucin hypersecretion is commonly observed in many respiratory diseases, such as, rhinitis, sinusitis, otitis media, nasal allergy, chronic bronchitis and cystic fibrosis.[1-4] Eighteen types of mucin genes have been

discovered to date: *MUC1*, [5] *MUC2*, [6] *MUC3*, [7] *MUC4*, [8] *MUC5AC*, [9] *MUC5B*, [10] *MUC6*, [11] *MUC7*, [12] *MUC8*, [13] *MUC9*, [14] *MUC10*, [15] *MUC11*, [16] *MUC12*, [16] *MUC13*, [17] *MUC15*, [18] *MUC16*, [19] *MUC17*, [20] and *MUC18*.[21] Of these, *MUC5AC* and *MUC5B* are generally recognized to be the major airway mucin because *MUC5AC* is highly expressed in the goblet cells of the human airway epithelium.[22-24] Moreover, *MUC5AC* gene expression is known to be regulated by oxidative stress [25] and retinoic acid.[26] In addition, *MUC5AC* is regulated by various inflammatory cytokines such as neutrophil elastase, [27] IL-9, [28] and IL-4.[29] Given that mucin hypersecretion is an uncontrolled mucin expression during inflammation, unveiling of signal transduction pathway for inflammatory cytokine-induced *MUC5AC* gene expression would give an important clue to the understand of airway mucus hypersecretion.

It is well documented that mitogen-activated protein (MAP) kinase pathways are thought to be most important in transmitting inflammatory signals from the cell surface to the nucleus.[30] After being triggered by growth factors, cytokines, UV rays, or other stress-inducing agents, a signal is delivered down the MAPKKK $\rightarrow$ MAPKK (in the cases of ERK, JNK, and p38, the signal is delivered through MEK1/2, MKK4/7, and MKK3/6, respectively) $\rightarrow$ to the MAP kinase cascade. The MAP kinases play a role in cell proliferation, differentiation, apoptosis, cytoskeletal remodeling, and the cell cycle.[31-36]

Mitogen- and stress-activated protein kinase 1 (MSK1) is a recently identified enzyme that is widely distributed in mammalian cells.[37-39] MSK1 is activated *in vitro* and *in vivo* by two different classes of MAP kinase, ERK and p38 MAP kinases.[37] Moreover, MSK1 is localized in the nuclei of stimulated or unstimulated cells [39] and two potential *in vivo* substrates are the cAMP response element binding protein (CREB) and the closely related activating transcription factor 1 (ATF1).[39]

Recently, reactive oxygen species are reported to increase the expression of the *MUC5AC* gene, by activating the ERK MAP kinase pathway, [25] and nontypeable *Haemophilus influenzae* (NTHi) was reported to regulate *MUC5AC* transcription via p38 MAP kinase in human epithelial cells.[40] In a study of *MUC2*, *Pseudomonas aeruginosa* was found to activate NF-κB through Ras-MAPK-pp90rsk, which led to the increased expression of MUC2, but p38 was not involved in this pathway.[41] However, the mechanism of *MUC5AC* gene expression during inflammation in normal airway epithelial cells, and the signal molecules involved, especially in the downstream signaling of MAP kinases have not yet been demonstrated.

Because *MUC5AC* hypersecretion during inflammation plays an important role in the pathogenesis of airway diseases, we hypothesized that major inflammatory cytokines, IL-1β and TNF-α up-regulate *MUC5AC* gene

expression by activating specific signal transduction pathways in airway epithelial cells. Here we show that two different MAP kinases, ERK and p38 MAP kinases, are essential for IL-1β- and TNF-α-induced *MUC5AC* gene expression in normal human nasal epithelial (NHNE) cells. We also show that MSK1 mediates the IL-1β- and TNF-α-induced phosphorylation of CREB and the transcription of *MUC5AC*. Furthermore, the cAMP response element (CRE) in *MUC5AC* promoter appears to be important for IL-1β- and TNF-α-induced *MUC5AC* gene expression in NCI-H292 cells. These pathways provide new insights into molecular mucus hypersecretion and may open up novel targets for therapeutic intervention.

II. MATERIALS AND METHODS

1. Materials

PD98059, SB203580 and anti-α-tubulin antibody were purchased from Calbiochem (San Diego, CA). Anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody, anti-phospho-p38 MAP kinase (Thr180/Tyr182) antibody, anti-phospho-SAPK/JNK MAP kinase (Thr183/Tyr185) antibody, anti-phospho-MSK1 (Thr581) antibody, and anti-phospho-CREB (Ser133) antibody were purchased from Cell Signaling (Beverly, MA). Plasmid encoding kinase-deficient MEK1 mutant (*pcDNA5-MEK1DN*) and p38 mutant (*pcDNA3-p38AGF*) were kindly provided by Dr. Jian-Dong Li (House Ear Institute, Los Angeles, CA) and Dr. Yoshiyuki Kuchino (National Cancer Center Research Institute, Saitama, Japan), respectively. Wide-type MSK1, N-and C-terminal kinase dead MSK1 mutant constructs were kindly provided by Dr. Dario Alessi (University of Dundee, Dundee, UK). Reporter construct, the 3.8-kb *MUC5AC* 5'-flanking region fused to a luciferase reporter gene, was kindly provided by Dr. Carol Basbaum (University of California, San Francisco, CA).

2. Cell Cultures.

The culture system used for the normal human nasal epithelial (NHNE) cells was as described previously.[42] 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)

supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin-streptomycin at 37 ℃ in a humidified chamber with 5% CO_2. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline and recultured in RPMI-1640 with 0.2% FBS.

3. RT-PCR

Total RNA was isolated using TRIzol (Invitrogen) from NCI-H292 cells treated with IL-1β (10 ng/ml) or TNF-α (10 ng/ml). cDNA was synthesized with random hexamers (Perkin Elmer Life Sciences and Roche Applied Science) using Moloney murine leukemia virus-reverse transcriptase (PerkinElmer Life Science). Oligonucleotide primers for PCR were designed based on the Genbank™ sequence of *MUC5AC* (Genbank™ accession number AJ001402, 5' primer CGACAACTACTTCTGCGGTGC; 3' primer GCACTCA-TCCTTCCTGTCGTT). The following PCR conditions used involved 35 cycles: denaturation at 94 ℃ for 30 sec, annealing at 60 ℃ for 30 sec, and polymerization at 72 ℃ for 30 sec. The oligonucleotide primers for β_2-microglobulin (used as a control gene for the RT-PCR) were designed based on the Genbank™ human sequence (Genbank™ accession number XM007650, 5' primer CTCGCGCTACTCTCTCTTTCTGG; 3' primer GCTTACATGTCTCG-ATCCCACTTAA). PCR parameters used involved 23 cycles as follows: denaturation at 94 ℃ for 30 sec, annealing at 55 ℃ for 30 sec, and polymerization at 72 ℃ for 30 sec. The PCR products were run in a 1.5%

agarose gel and visualized with ethidium bromide under a transilluminator.

4. Real-time Quantitative PCR

Primers and probes were designed with PerkinElmer Life Sciences Primer Express® software and purchased from PE Biosystems. Commercial reagents (TaqMan PCR Universal PCR Master Mix, PerkinElmer Life Sciences) and conditions according to the manufacturer's protocol were applied. An amount of 1 μg of cDNA (reverse transcription mixture) and oligonucleotides at a final concentration of 800 nM of primers and 200 nM of TaqMan hybridization probe were analyzed in a 25 μl volume. The following primers and TaqMan probes were used: *MUC5AC*, forward 5'-CAGCCACGTCCCCTTCAATA-3' and reverse 5' ACCGCATTTGGGCATCC-3' and Taqman probe 6FAM-CCACCTCCGAGCCCGTCAC- TGAG-TAMRA. β_2M, forward 5'-CGCTCC-GTGGCCTTAGC-3' and reverse 5'-GAGTACGCTGGATAGCCTCCA-3' and Taqman probe 6FAM-TGCTCGCGCTACTCTCTCTTTCTGGC-TAMRA. Real-time RT-PCR was performed on a PerkinElmer Life Sciences ABI PRISM® 7700 Sequence Detection System (Foster City, CA). The thermocycler (ABI PRISM® 7700 Sequence Detection System) parameters were 50 ℃ for 2 min, 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 sec and 60 ℃ for 1 min. All reactions were performed in triplicate. Relative quantity of *MUC5AC* mRNA was obtained using comparative CT method and was normalized using β_2M as an endogenous control.

5. Western Blot Analysis

NCI-H292 cells were grown to confluence in 6 well plates. After 15 or 45 min treatment with IL-1β or TNF-α, respectively, the cells were lysed with 2x 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% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA). Membranes were blocked with 5% skim milk in Tris-buffered saline [TBS; 50 mM Tris-Cl (pH 7.5), 150 mM NaCl] for 2 hr at room temperature. This blot was then incubated overnight with primary antibody in TTBS (0.5% Tween 20 in TBS). After washing with TTBS, the blot was further incubated for 45 min at room temperature with anti-rabbit or anti-mouse antibody (Cell Signaling) in TTBS, and then visualized by using the ECL system (Amersham Biosciences, Piscataway, NJ).

6. *In vitro* p38 kinase assay

p38 Kinase activity was measured using a p38 MAP kinase assay kit (Cell Signaling) according to the manufacturer's instructions. Briefly, confluent cells were rendered quiescent for 24 hr and then incubated with or without 20 μM SB203580 for 1 hr prior to being stimulated with IL-1β or TNF-α for 15 min. Cells lysates were scraped off the dish with 500 μl lysis buffer and 1 mM PMSF, sonicated 4 times for 5 sec each on ice and centrifuged for 10 min at 4 ℃. Supernatants then transferred to a new tube, and 400 μg of the cell lysates and 20 μl of immobilized phospho-p38 MAP kinase monoclonal antibody were

incubated with gentle rocking overnight at 4 ℃. The pellet was washed twice with lysis and kinase buffer and then resuspended in kinase buffer containing 200 μM ATP and 2 μg of activating transcription factor 2 (ATF2) fusion protein. It was then incubated for 30 min at 30 ℃, and immunoblotted with phospho-ATF2 antibody.

7. Preparation of Inducible Dominant-Negative Mutant Stable Cell Lines

Plasmid encoding the kinase-deficient MEK1 mutant (*pcDNA5-MEK1DN*) was cut with *Bam*H1, and ligated with *pBluescript* (Stratagene, La Jolla, CA). This clone was cut with *Hin*dIII, filled in with Klenow, and cut with *Sac*II (Promega), and then ligated with *pTRE* vector (Clontech, Palo Alto, CA). Plasmid encoding kinase-inactive p38 mutant (*pcDNA3-p38AGF*) was cut with *Bam*H1, filled in with Klenow, cut with *Xba*I (Promega, Madison, WI), and then ligated with *pTRE* vector. NCI-H292 cells were cotransfected with *pTet-off* (Clontech) regulation vector and *pTRE-p38DN* or *pTRE-MEK1DN* (1:20 ratio of regulation vector to expression vector) using FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN), following the procedure recommended by the manufacturer. Stably transfected cell lines were selected with 200 μg/ml G418 (Calbiochem), and the medium was replaced with G418 and doxycycline every 3 days.

8. 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% NP-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 ℃ and the supernatants were stored at -70 ℃. For EMSA, oligonucleotides corresponding to the consensus CRE sequences (5'-AGAGATTGCCTGACGTCAGAGAGC-TAG-3'), CRE-specific sequences in the *MUC5AC* promoter region -878 to -871 (5'-AGAGATTGCCTGACTTGAAGAGCTAG-3'), and the CRE mutant sequence (5'-AGAGATTGCCTGACTGACAGAGCTAG-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(dI-dC)]. DNA-nuclear protein complexes were separated from the DNA probe by electrophoresis through 5% nondenaturing polyacrylamide gels in

0.5X Tris Borate EDTA (TBE) buffer. Supershift experiments were conducted using 2 μl of anti-phospho-CREB antibody. The gel was dried and autoradiographed using an intensifying screen at –70 ℃.

9. Plamids, Transient Transfection and Luciferase Assay

Cells were transiently transfected with plasmids containing wide-type MSK1, N-terminal kinase dead MSK1 mutant (D195A), C-terminal kinase dead MSK1 mutant (D565A), CREB DN (S133A), and reporter constructs, the *MUC5AC* 5'-flanking region fused to a luciferase reporter gene using FuGENE6 transfection reagent (Roche Applied Science) according to the manufacturer's instructions. Deletion mutants covering promoter regions of *MUC5AC* were generated by PCR using pairs of primers bearing specific restriction sites at their 5' and 3' ends (Table I) and then were constructed in promoterless pGL3 basic vector. Cells were incubated for 48 hr, 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 the transfection efficiency of each sample.

III. RESULTS

1. IL-1β and TNF-α Can Induce the Gene Expression of *MUC5AC* through ERK and p38 MAP kinases Signaling in NHNE cells.

To determine whether IL-1β and TNF-α can induce *MUC5AC* gene expression within NHNE cells, we carried out RT-PCR after treatment with IL-1β or TNF-α for 24 hr. The results showed that *MUC5AC* mRNA was significantly increased after treatment with IL-1β or TNF-α in NHNE cells (Fig. 1A). No corresponding change was found in the expression of internal control, β_2-microglobulin. As a next step, to determine which MAP kinase signal pathway is activated within NHNE cells stimulated by IL-1β or TNF-α, we performed a Western blot analysis using phospho-specific antibodies. ERK and p38 MAP kinases were maximally activated at 15 min, and this effect decreased at 45 min (Fig. 1B). However, no change was detected in the activation of JNK. A549 cells were used as a positive control for JNK activation. It thus appeared that stimulation by IL-1β and TNF-α induced the ERK and p38 MAP kinases pathways in NHNE cells. In order to investigate the possible involvement of ERK and p38 MAP kinases in IL-1β- and TNF-α-induced *MUC5AC* gene expression, 20 μM PD98059, specific MEK1/2 inhibitor, or 20 μM SB203580,

p38 inhibitor, were applied before treatment with IL-1β and TNF-α. The Western blot and *in vitro* kinase assays showed that PD98059 and SB203580 clearly inhibited ERK and p38 MAP kinases, respectively, in NHNE cells (Fig. 2A and 2B). Under this experimental condition, we checked the expression level of *MUC5AC* by performing real-time quantitative PCR analysis. These results showed that pretreatment with PD98059 or SB203580 for 1 hr inhibited *MUC5AC* gene expression (Fig. 2C and 2D). Interestingly, the inhibition of either ERK or p38 MAP kinase pathway inhibited *MUC5AC* mRNA in NHNE cells. Thus, the activation of ERK and p38 MAP kinases appeared to be closely related to the signaling pathways activated by IL-1β or TNF-α. We next examined whether there may be a cross-talk between ERK and p38 MAP kinases. We found that pretreatment of SB203580 suppressed the IL-1β- or TNF-α-induced ERK, while pretreatment of PD98059 did not affect the IL-1β- or TNF-α-induced p38 in NCI-H292 cells (Figs. 2E and 2F). These results suggest that p38 MAP kinase can mediate IL-1β- or TNF-α-induced activation of ERK as a cross-talker.

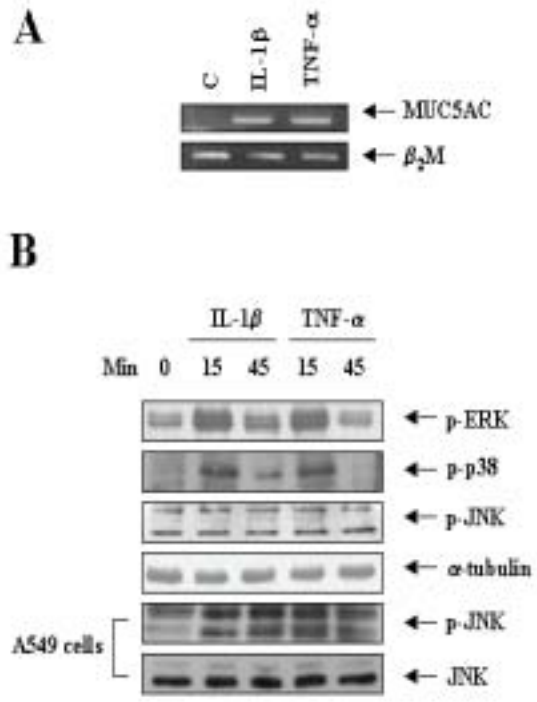

Fig. 1. Effect of IL-1β and TNF-α on *MUC5AC* gene expression in NHNE cells. Confluent cells were treated with IL-1β (10 ng/ml) or TNF-α (10 ng/ml) for 24 hr and cell lysates were harvested for RT-PCR (***A***). C, control. β_2-microglobulin was employed as an internal control. Confluent cells were treated with IL-1β (10 ng/ml) or TNF-α (10 ng/ml) for 15 or 45 min and cell lysates were harvested for Western blot analysis. Representative Western blots (***B***) using phospho-specific antibodies showed transient activation of ERK and p38 but not of JNK, the maximum effect is at 15 min. The figures shown are representative of three independent experiments.

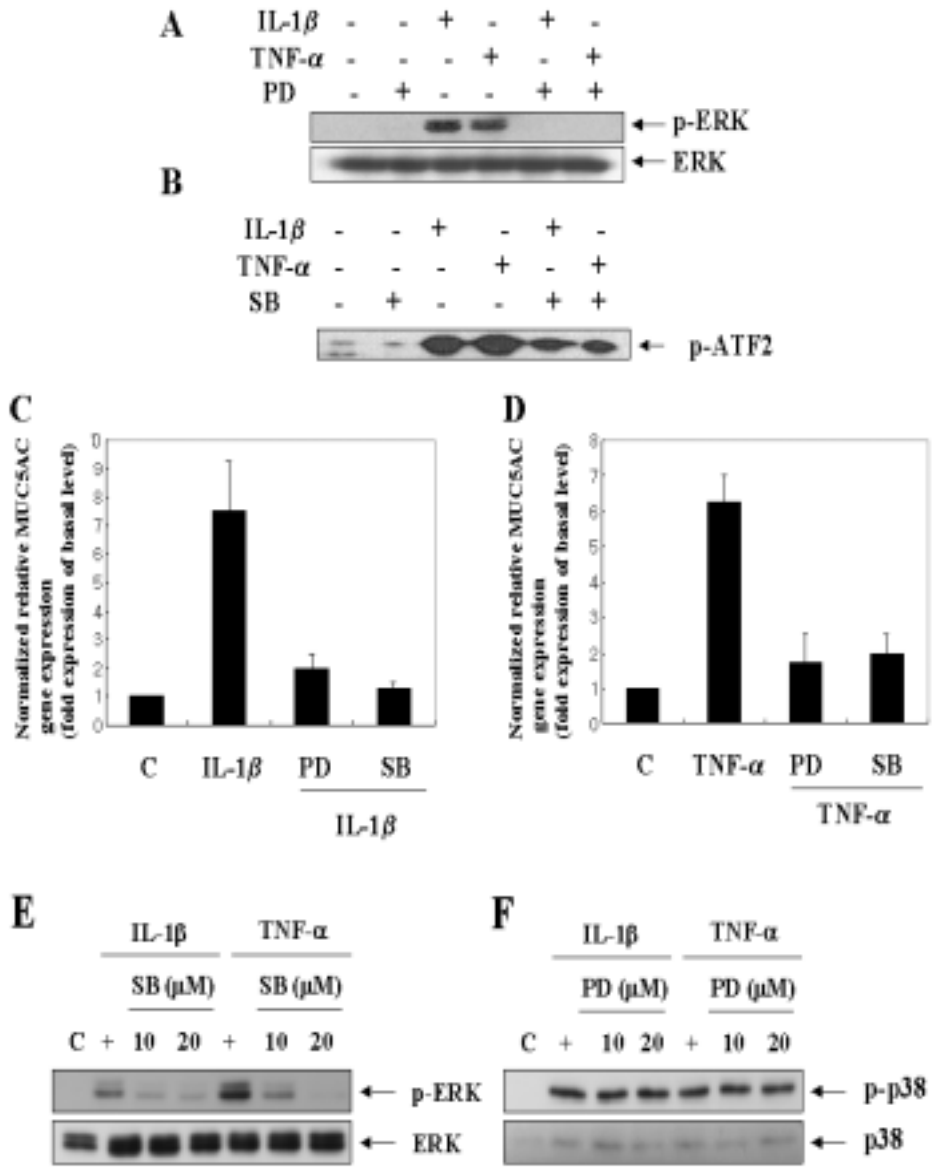

Fig. 2. Effect of ERK and p38 MAP kinases on *MUC5AC* gene expression in NHNE cells. Confluent cells were pretreated for 1 hr with 20 μM PD98059 or

20 μM SB203580, and then stimulated for 15 min with IL-1β or TNF-α prior to collection of total proteins for kinase assays. Representative kinase assays show the inhibition of phosphorylation of ERK by PD98059 (PD) (***A***) and ATF2 as an exogenous substrate (***B***) with p38 MAP kinase immunoprecipitated from IL-1β- or TNF-α-treated cells, and the inhibition of p38 MAP kinase activation by SB203580 (SB). Cells were pretreated and stimulated for 24 hr with IL-1β or TNF-α prior to collection of total RNA for real-time quantitative PCR of *MUC5AC* (***C and D***). C, control. The figures shown are representative of three independent experiments.

2. Both ERK and p38 MAP kinases are Essential for IL-1β- or TNF-α-induced *MUC5AC* Gene Expression.

When the same experiments (Fig. 1 and 2) were performed using NCI-H292 cells, human lung mucoepidermoid carcinoma cell line, the results were the same in the NCI-H292 cells as in the normal cells (data not shown). In order to confirm the significance of ERK and p38 kinases upon cellular level of *MUC5AC* gene expression, cells stably expressing dominant-negative (DN) mutant under control of Tet-off system were generated. After removing doxycycline to induce MEK1DN, stimulation with IL-1β or TNF-α for 15 min, decreased the phosphorylation of ERK (Fig. 3A). However, no change in ERK expression was observed. Real-time quantitative PCR showed a decrease in *MUC5AC* mRNA after 24 hr for IL-1β and TNF-α. In a similar way, we investigated the role of p38 MAP kinase on IL-1β- or TNF-α-induced *MUC5AC* gene expression using p38DN. The p38 DN mutant was generated by replacing Thr180 and Tyr182 by Ala and Phe, respectively.[43] This inactive form of p38 MAP kinase binds endogenous substrates, thereby inhibiting signaling by the endogenous p38 MAP kinase pathway. An *in vitro* kinase assay showed that the activation of p38 MAP kinase in this mutant stable cell lines was decreased following IL-1β and TNF-α stimulation (Fig. 3B). Under this condition, *MUC5AC* mRNA was decreased 24 hr after treatment with IL-1β and TNF-α. These results showed that ERK and p38 MAP kinases are essential for IL-1β- and TNF-α-induced *MUC5AC* gene expression in NCI-H292 cells.

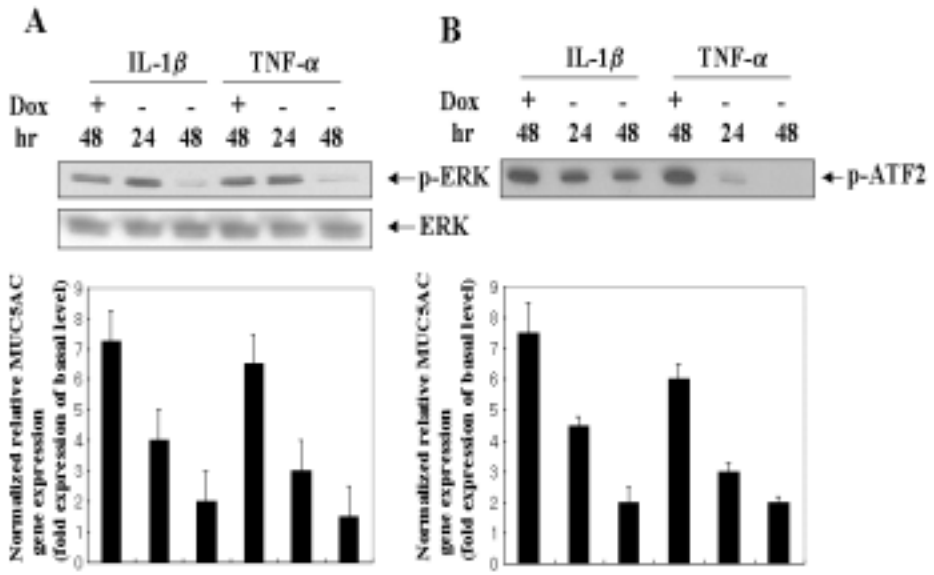

Fig. 3. ***MUC5AC*** **gene expression in MEK1 or p38 dominant-negative mutant stable cell lines**. Confluent, quiescent MEK1 (***A***) and p38 (***B***) dominant-negative mutant stable cells were preincubated for the indicated times to induce dominant-negative mutant protein by removed doxycycline (Dox) and then stimulated for 15 min with IL-1β or TNF-α prior to Western blotting and then for 24 hr with IL-1β or TNF-α prior to real-time quantitative PCR. The figures shown are representative of three independent experiments. C, control.

3. IL-1β- or TNF-α-induced Activation of MSK1 is Mediated by both ERK and p38 MAP kinases.

To determine which molecules are involved in the downstream signaling of the ERK and p38 MAP kinases within the signal pathway of *MUC5AC* gene expression induced by IL-1β or TNF-α, we investigated the phosphorylation of MSK1 by phospho-specific antibody. MSK1 is widely distributed in mammalian cells and can be activated by MAPK/ERK and SAPK2a/p38.[37-39] The result showed that the phosphorylation of MSK1 by IL-1β or TNF-α reached a maximum at 30 min and decreased at 60 min after IL-1β and TNF-α stimulation (Fig. 4A and 4B). Pretreatment with 20 μM PD98059 and/or 20 μM SB203580 inhibited IL-1β- or TNF-α-induced MSK1 phosphorylation (Fig. 4C and 4D), indicating that MSK1 is regulated by ERK and/or p38 MAP kinase(s). Furthermore, to determine whether MSK1 influences MAP kinases, cells were transfected with DNA expression constructs encoding a mutant MSK1 (NT-KD) that the N-terminal kinase domain was inactivating by a point mutation and a further mutant (CT-KD) that the C-terminal kinase domain was inactivating.[39] Two MSK1 mutants did not affect IL-1β- and TNF-α-induced MAP kinases activation (Fig. 4E), indicating that MSK1 appears to be controlled by MAP kinases. These results showed that MSK1 acts as a downstream signaling mediator of ERK and p38 MAP kinases.

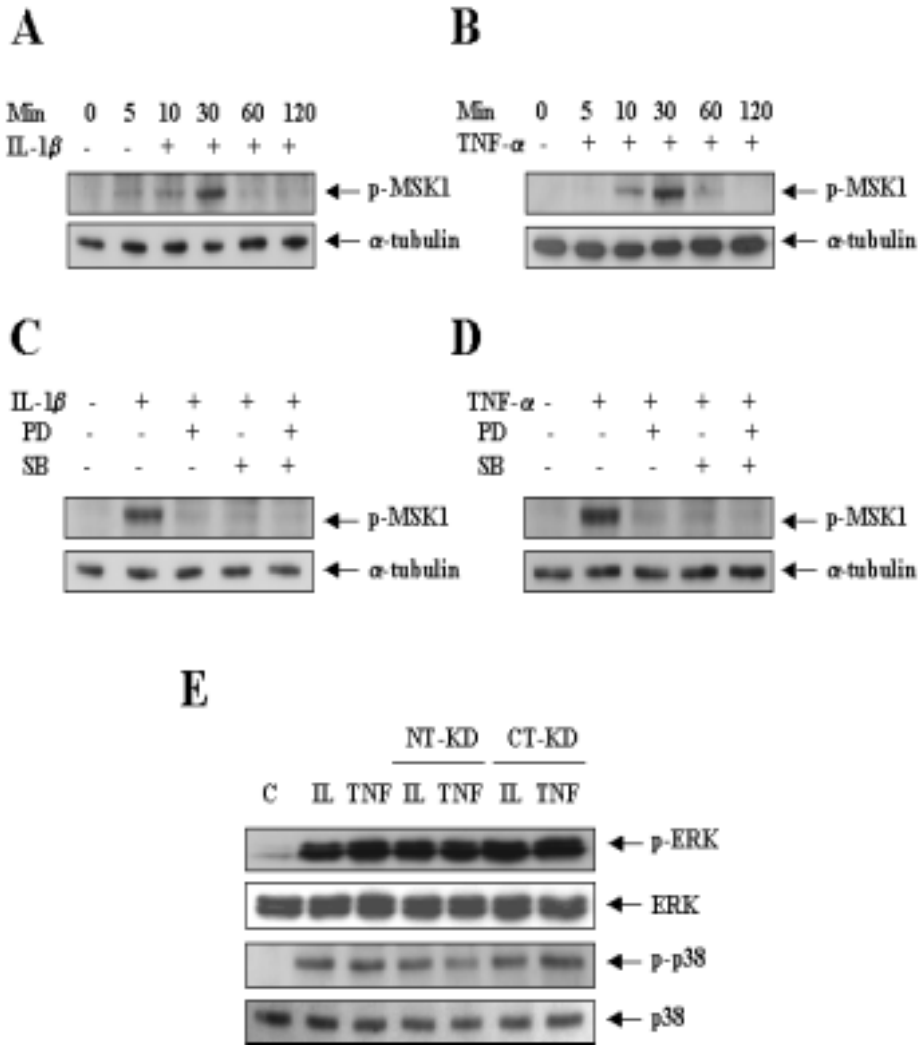

Fig. 4. Effect of ERK and p38 MAP kinases on IL-1β- or TNF-α-induced activation of MSK1. Confluent, quiescent cells were stimulated for the indicated times with IL-1β (***A***) or TNF-α (***B***), and then total proteins were

collected for Western blot analysis. In other experiments, the cells were pretreated for 1 hr with 20 μM PD98059 and/or 20 μM SB203580 and then the cells were then stimulated for 30 min with IL-1β (***C***) or TNF-α (***D***). The cells were transiently transfected with NT-KD or CT-KD MSK1 constructs and stimulated with IL-1β or TNF-α for 15 min (***E***) prior to Western blot analysis. C, control. The figures shown are representative of three independent experiments.

4. Effects of MSK1 and CREB on IL-1β- and TNF-α-induced *MUC5AC* Gene Expression.

To examine the role of MSK1 on the induction of *MUC5AC* gene expression, cells were transiently transfected with DNA expression constructs encoding wild-type MSK1 (W/T), NT- or CT-KD MSK1 mutant. IL-1β or TNF-α-induced *MUC5AC* gene expression increased in cells transfected with W/T MSK1, whereas overexpression of NT- or CT-KD MSK1 markedly suppressed IL-1β or TNF-α-induced *MUC5AC* gene expression (Fig. 5A and 5B). These results showed that MSK1 appears to be closely related in the *MUC5AC* gene expression by IL-1β and TNF-α.

Because MSK1 is currently the best candidate for the mediation of cytokine-induced CREB phosphorylation at Ser133, [37-39] we investigated the possible implication of CREB in IL-1β- or TNF-α-induced *MUC5AC* gene expression. To examine whether IL-1β or TNF-α can induce the phosphorylation of endogenous CREB via MAP kinases and MSK1 in airway epithelial cells, we performed Western blot using phospho-specific CREB (Ser133) antibody. A transient phosphorylation of CREB was observed upon the stimulation with IL-1β and TNF-α, reaching the maximum peak at 30 min (Fig. 6A and 6B). In addition, Pretreatment with PD98059 and/or SB203580 or NT- or CT-KD MSK1 remarkably inhibited the phosphorylation of CREB (Fig. 6C and 6D).

Next, to determine whether CREB plays a role in *MUC5AC* gene expression, we used forskolin (an activator of adenylate cyclase) and 3-isobutyl-1-methylxanthine (IBMX; an inhibitor of AMP phosphodiesterase). A transient phosphorylation of CREB was observed upon the stimulation with forskolin and IBMX, reaching the maximum peak at 10 min (Fig. 7A). The cAMP pathway-induced CREB phosphorylation increased *MUC5AC* gene expression (Fig. 7B). Furthermore, IL-1β- and TNF-α-induced *MUC5AC* gene expression was significantly suppressed in cells transfected with plasmid encoding mutant CREB (*pCREB S133A*) (Fig. 7C). These findings suggested that the activation of MSK1 and CREB is essential for IL-1β- and TNF-α-induced *MUC5AC* gene expression via ERK and p38 MAP kinases.

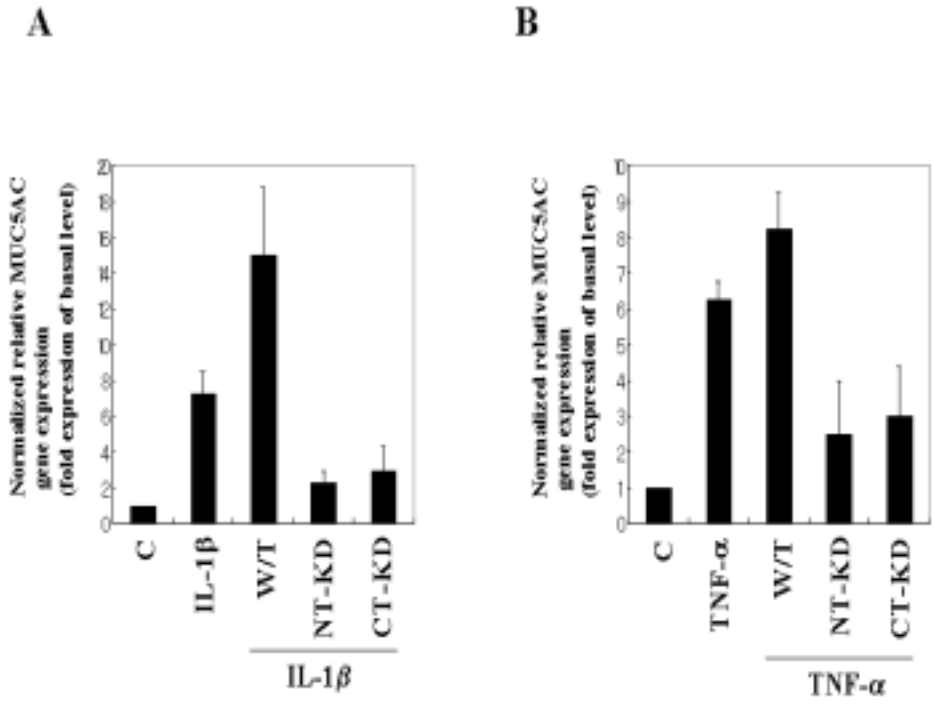

Fig. 5. Effect of MSK1 on IL-1β- and TNF-α-induced *MUC5AC* gene expression. Cells were transiently transfected with DNA constructs expressing wide-type MSK1 (W/T), N-terminal kinase dead (NT-KD) MSK1 and C-terminal kinase dead (CT-KD) MSK1. They were stimulated for 24 hr with IL-1β (***A***) or TNF-α (***B***). The cells were lysed and performed real-time quantitative PCR. C, control. The figures shown are representative of three independent experiments.

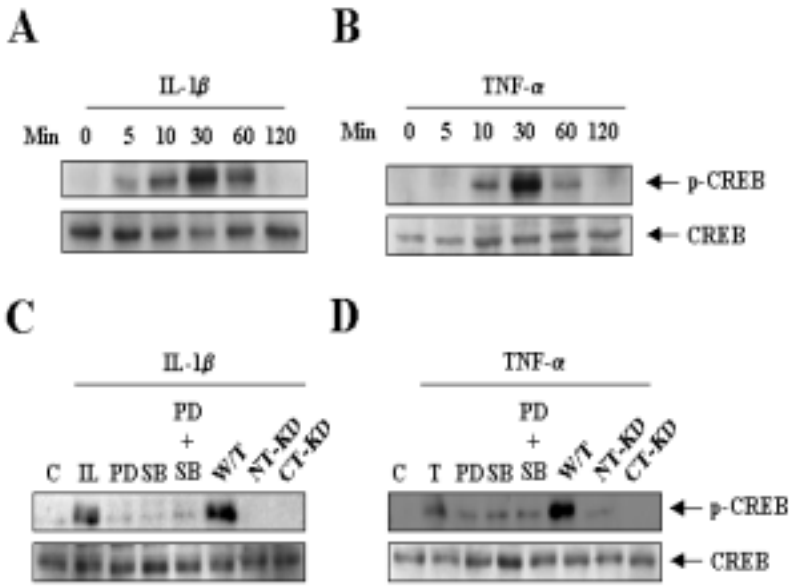

Fig. 6. IL-1β and TNF-α can induce the activation of CREB via MAP kinases and MSK1. Confluent, quiescent cells were stimulated for the indicated times with IL-1β (***A***) or TNF-α (***B***), and then total proteins were collected for Western blot analysis. In other experiments, the cells pretreated for 1 hr with 20 μM PD98059 or/and 20 μM SB203580 and the transfected cells with NT-KD or CT-KD MSK1 constructs were stimulated for 30 min with IL-1β (***B***) or TNF-α (***D***) prior to Western blot analysis. C, control. The figures shown are representative of three independent experiments.

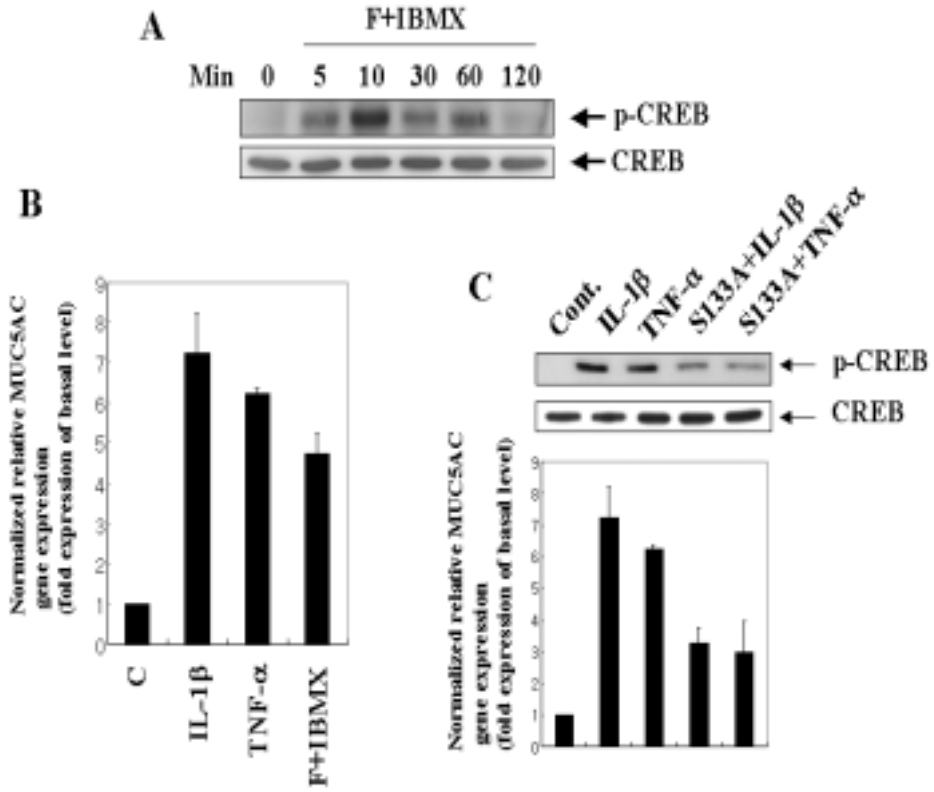

Fig. 7. Effect of CREB on IL-1β- and TNF-α-induced *MUC5AC* gene expression. Confluent, quiescent cells were stimulated for the indicated times with both 20 μM forskolin and 10 μM IBMX, and then total proteins were collected for Western blot (***A***). Cells were stimulated for 24 hr with IL-1β, TNF-α, or both 20 μM forskolin and 5 μM IBMX, and the total RNA were then subjected to real-time quantitative PCR (***B***). The cells were transiently

transfected with mutant CREB (*pCREB S133A*) constructs and stimulated with IL-1β or TNF-α for 30 min prior to Western blot analysis (***C, upper panel***) and for 24 hr prior to real-time quantitative PCR (***C, lower panel***). C, control. The figures shown are representative of three independent experiments.

5. Identification of the Binding Complex between CREB and CRE in Response to IL-1β and TNF-α.

To analyze the DNA binding activity of IL-1β- and TNF-α-activated CREB, we performed EMSA using nuclear extracts from NCI-H292 cells after IL-1β (A) or TNF-α (B) treatment. As shown in Fig. 8A and 8B, the activity of consensus CRE oligonucleotide (CREc) and *MUC5AC* specific CRE (CREs) remarkably increased in response to IL-1β or TNF-α, but not by mutant CRE of *MUC5AC* promoter (CREm) oligonucleotide. To distinguish any specific CRE-binding complexes, competition and supershift analysis were performed using 50-fold excesses of non-radiolabeled (cold) CREs oligonucleotide and anti-phospho-CREB antibody, respectively. The specific band was found to be selectively inhibited by the specific CRE competitor and was supershifted by anti-phospho-CREB antibody. These results indicated that activated CREB binds to a *cis*-acting element, CRE, in the *MUC5AC* promoter.

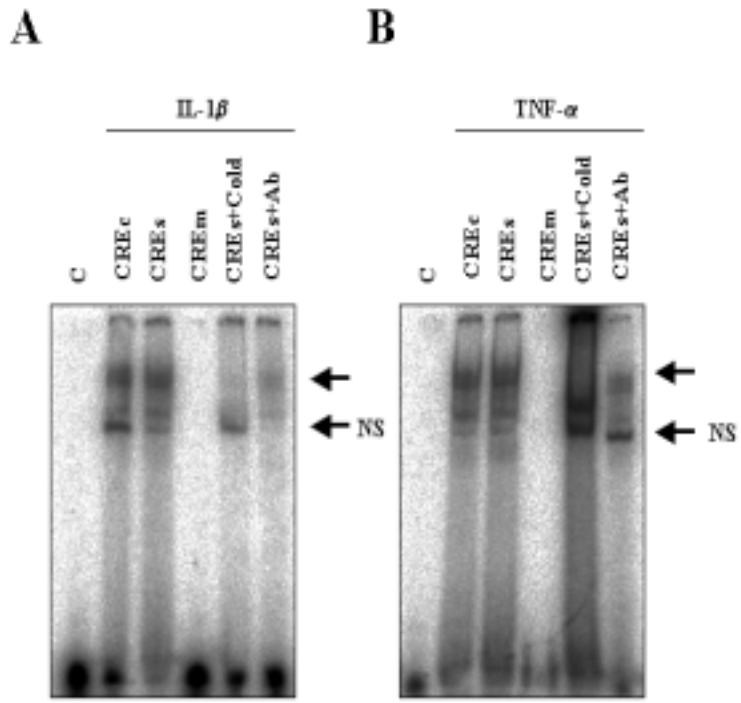

Fig. 8. IL-1β- or TNF-α-induced nuclear binding of CRE. Confluent, quiescent cells were stimulated for 1 hr with IL-1β (***A***) or TNF-α (***B***). Nuclear protein extracts from IL-1β- or TNF-α-treated NCI-H292 cells were subjected to EMSA. Nuclear proteins were incubated with CREc, CREs, CREm, 50-fold excess of cold probe or anti-phospho-CREB antibody before EMSA. The labeled nuclear proteins were separated by elecrophoresis on 5% polyacrylamide gels, and the gels were dried and exposed to autoradiography at

–70 °C overnight.

C, control; Ab, antibody; CREc, consensus CRE; CREs, *MUC5AC*-specific CRE; CREm, *MUC5AC*-mutant CRE; NS, non-specific.

6. Identification of IL-1β- and TNF-α-responsive Regions within *MUC5AC* promoter.

Cells were then transiently transfected with the various deletion mutants and treated with IL-1β (40 ng/ml) or TNF-α (40 ng/ml) for 24 hr, respectively. As shown in Fig. 9A, IL-1β and TNF-α selectively increased luciferase activity of -929/+4 region of *MUC5AC* promoter. No effect was seen on fragments covering -1376/+4, -776/+4 and -486/+4 regions, indicating that the -929/-776 region of *MUC5AC* promoter may be necessary to observe a response to IL-1β or TNF-α. To further know whether CRE within -929/-776 region of *MUC5AC* promoter, identified using the TRANSFAC 4.0 data base, critically acts as *cis*-element, cotransfection with plasmid expression construct encoding mutant CREB was performed to study its effect on *MUC5AC* transcription activity. CREB DN suppressed luciferase activity of -929/+4 region of *MUC5AC* promoter (Fig. 9B). Moreover, we examined whether activation of CRE is required for IL-1β- and TNF-α-induced *MUC5AC* transcription by performing selective mutagenesis of the CREB-binding site. As a shown in Fig. 9C, mutant constructs M1, M2 and M3 abolished responsiveness of wild-type *MUC5AC* promoter construct (Fig. 9C). These results showed that CRE in the regulatory region of *MUC5AC* promoter was critical for the up-regulation of the transcriptional activity of *MUC5AC* induced by IL-1β or TNF-α.

Position of the DNA fragment	Orientation	Oligonucleotide used for PCR
-929	S	CGC *GAGCTC* GTC CAG AGG GTA CTG AGC
-776	S	CGC *GAGCTC* CAT TTG CCT GGA GGC TGC
-486	S	CGC *GAGCTC* CTCCCTCCC AGG CAG CCA
+4	AS	CGC *AAGCTT* GAG GGA CCC AAG GTG GCA

Table I

Sequence of the pairs of oligonucleotides used in PCR to produce deletion mutant covering MUC5AC 5'-flanking region.

*Sac*I (GAGCTC) and *Hin*dIII (AAGCTT) sites were added at the end of the primers to direct subcloning and were italicized and underlined. Positions of the DNA fragments relative to the published transcription initiation sites (Ref. 50) are indicated. S, sense; AS, antisense

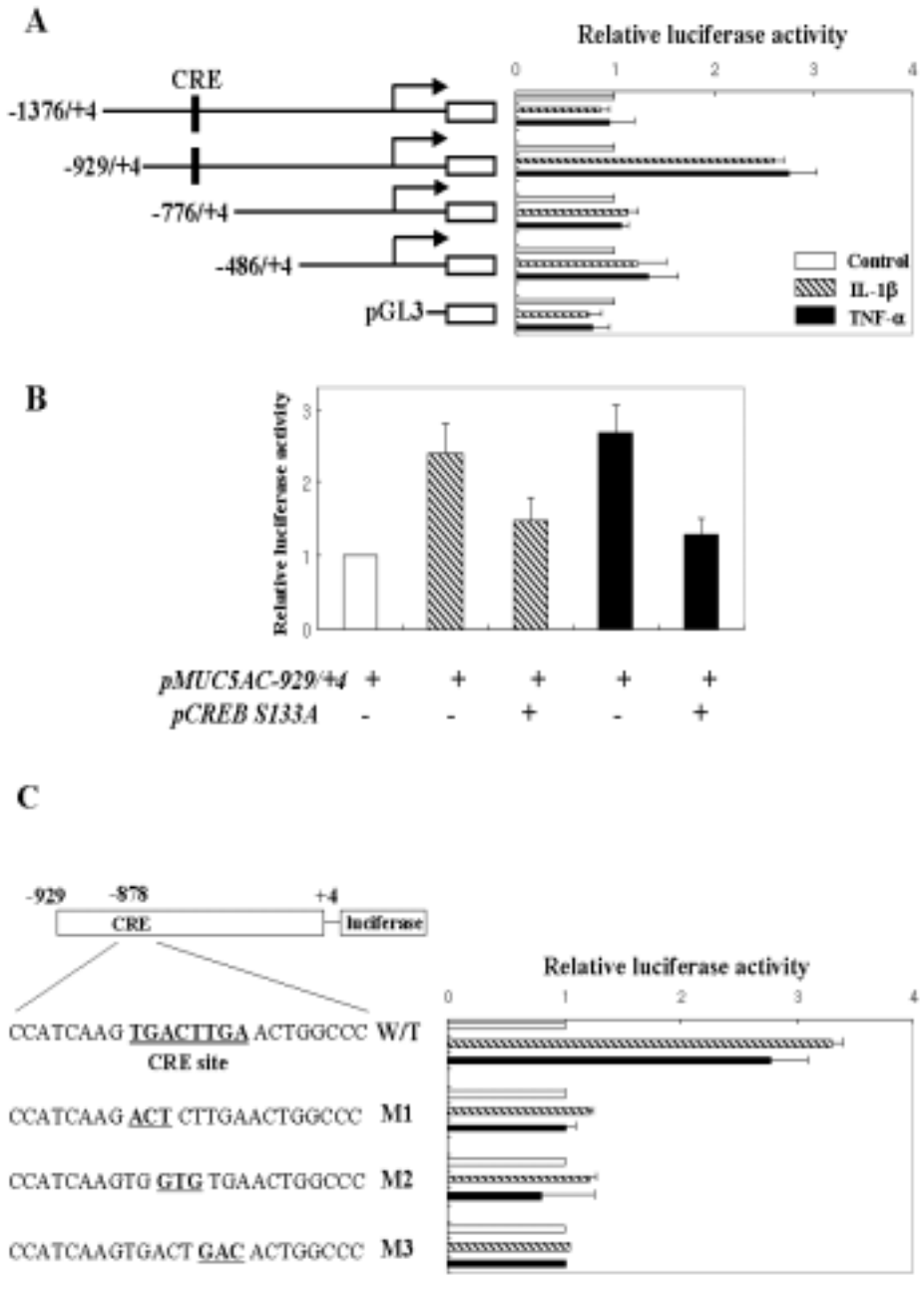

Fig. 9. IL-1β- and TNF-α-induced activation of CRE-mediated *MUC5AC*

transcription via the *cis*-acting regulatory CRE motif. NCI-H292 cells were transiently transfected with various *MUC5AC* promoter luciferase reporter constructs and stimulated with IL-1β (40 ng/ml) and TNF-α (40 ng/ml) for 24 hr. Luciferase activity was then assessed in IL-1β or TNF-α-treated and -untreated cells (***A***). Cells were cotransfected with a dominant-negative mutant of CREB and reporter construct of -929/+4 region of *MUC5AC* promoter (***B***) and transfected with *MUC5AC* promoter construct containing various mutated CRE site as indicated (***C***). The luciferase activities were displayed after correction for transfection efficiency using the β-galactosidase activity of the cell lysates to standardize the values. The values shown are means ± S.D. of experiments performed in triplicate.

IV. DISCUSSION

Mucin hypersecretion causes many clinical problems, such as rhinorrhea, nasal stuffiness, and sputum in the respiratory tract. It has been reported that *MUC5AC* is the major mucin in human airways.[22-24, 44] The mechanism of the regulation of *MUC5AC* secretion by inflammatory cytokines in airway is very important, and the understanding of this mechanism may offer new therapeutic strategies for the inhibition of airway mucus hypersecretion.

The molecular mechanism by which *MUC5AC* is up-regulated by IL-1β and TNF-α remains poorly understood. In the present study, we undertook to reveal the related mechanism of IL-1β and TNF-α in the up-regulation of *MUC5AC* gene expression in normal human airway epithelial cells.

The fact that more than one MAP kinase may be necessary for the IL-1β- and TNF-α-induced *MUC5AC* gene expression in NHNE and NCI-H292 cells is an interesting finding of the present study (Fig. 2 and 3). Although *MUC5AC* is regulated by various inflammatory cytokines such as neutrophil elastase, [27] IL-9, [28] and IL-4, [29] it was not shown which mechanisms are essential for cytokines-induced *MUC5AC* gene expression. Recently, Takeyama *et al.* reported that epidermal growth factor (EGF) increased *MUC5AC* gene

expression via ERK MAP kinase, but not p38 MAP kinase in NCI-H292 cells.[25] Moreover, Wang *et al.* showed that NTHi regulated *MUC5AC* transcription via p38 MAP kinase, but did not mediate ERK MAP kinase.[40] In the present study, we showed that both ERK and p38 MAP kinase, but not JNK signaling, are essential for IL-1β- and TNF-α-induced *MUC5AC* gene expression. These suggest that the signaling pathways leading to *MUC5AC* gene expression are distinct, depending on the type of stimuli and cell lines used. We do not yet know how both pathways intervene in the activation of cytokine-induced *MUC5AC* gene expression. Recently, TNF-α-induced matrix metalloproteinase (MMP)-1 and MMP-3 gene expression is known to be regulated through AP-1-dependent transcriptional activation via ERK pathway and AP-1-independent enhancement via p38 MAPK by mRNA stabilization in human skin fibroblast.[44] Interestingly, TNF-α has an effect on the stability of *MUC5AC* mRNA in NCI-H292 cells.[45, 46] Thus, taken together, it is conceivable that the intracellular signaling coordination controlled by ERK in combination with p38 MAP kinase may be essential for IL-1β- and TNF-α-induced *MUC5AC* gene expression.

To date, signal molecules involved in the downstream signaling of MAP kinases for *MUC5AC* gene expression have not been yet demonstrated. The role of MSK1 and CREB in the downstream signaling of MAP kinases in the IL-1β- and TNF-α-induced *MUC5AC* gene expression in airway epithelial cells is a

major finding of the present study. MSK1 is known to be regulated by MAPK/ERK and SAPK2a/p38 and is currently the best candidate for the mediation of cytokine-induced CREB phosphorylation at Ser133.[37-39, 47] Although CREB activation by MSK1 has been established by previous studies in other cells, [37-39] it has remained unclear in airway epithelial cells. In addition, little is known about the involvement of CREB in *MUC5AC* gene expression. In this study, our results show that CREB activation is involved in the downstream signaling of MAP kinases and MSK1 for IL-1β- and TNF-α-induced *MUC5AC* gene expression.

Interestingly, *MUC5AC* mRNA was inhibited in *pCREB S133A* transfected cells treated by IL-1β and TNF-α, and treatment with forskolin and IBMX activated the phosphorylation of CREB and increased *MUC5AC* gene expression (Fig. 7A and 7B). These suggest that CREB may be a transcription factor for IL-1β-and TNF-α-induced *MUC5AC* gene expression. However, the increase of *MUC5AC* expression by forskolin and IBMX was less than that induced by IL-1β and TNF-α, suggesting that activation by CREB alone is insufficient to induce the full expression of *MUC5AC*. This result suggests that transcription factor, other than CREB, may be required for full expression of IL-1β- and TNF-α-induced *MUC5AC* gene expression. Previously, *P. aeruginosa*-induced *MUC2* expression is found to be regulated by NF-κB in NCI-H292

cells.[41] In fact, we could find the putative NF-κB binding site at -273 and -956 in *MUC5AC* promoter.[48] Therefore, NF-κB may also regulate cytokine-induced *MUC5AC* gene expression with the cooperation of CREB. This suggestion was further supported by our recent finding that IL-1β or TNF-α can initiate IκB degradation in NHNE and NCI-H292 cells and that pretreatment of caffeic acid phenethyl esther (CAPE), which is known to specifically block the translocation of p65 without affecting IκBα degradation, [41] inhibits *MUC5AC* gene expression induced by IL-1β (data not shown). In fact, Gerritsen *et al.* reported that p300 and CREB-binding protein (CBP) act as coactivators of p65 transactivation and may play an important role in the cytokine-induced expression of various immune and inflammation genes.[49] Furthermore, Perrais *et al.* reported that transcription factor Sp1 is essential for EGF-and TGF-α-mediated *MUC5AC* up-regulation.[50] Taken together, these findings suggest that CREB may interact directly or indirectly with other transcription factor(s) and that non-DNA binding transcriptional coactivators, such as p300 and CBP, which were thought to function as bridging proteins between DNA-binding transcription factors and the basal transcription factors, play a role as integrators of diverse signaling pathways in the *MUC5AC* gene expression.

Whereas CREB has recently emerged as potent regulator of mucins (*MUC2*, *MUC5AC*, *MUC5B* and *MUC6*), gene expression in the p15 arm of

chromosome 11 (11p15), [51] and cholera toxin A subunit (CTA), an activator of cAMP-dependent protein kinase, activates transcription of *MUC5B* promoter, [52] little is known about the involvement of CRE in *MUC5AC* transcription. Our results showed that -929/+4 region of *MUC5AC* promoter was sufficient to get a response to IL-1β or TNF-α and that CRE in -878 region of *MUC5AC* promoter was critical for the up-regulation of the transcriptional activity of *MUC5AC* induced by IL-1β or TNF-α. However, Perrais *et al.* reported that TNF-α did not have any significant effect of activity of -1366/+4 region of *MUC5AC* promoter, which was in accordance with our results in -1376/+4 region of *MUC5AC* promoter.[50] These results suggest that TNF-α-responsive repressor(s) or negative regulatory element (NRE) that represses inherent basal and cAMP-inducible promoter activity may be located in -1366/-929 region of *MUC5AC* promoter.[53] Thus, it seems necessary to explore further the involvement of IL-1β- or TNF-α-responsive repressor(s) or NRE in IL-1β- or TNF-α-induced *MUC5AC* transcription.

V. CONCLUSION

In this study, because *MUC5AC* hypersecretion during inflammation plays an important role in the pathogenesis of airway diseases, we examined that major inflammatory cytokines, IL-1β or TNF-α up-regulate *MUC5AC* gene expression by activating specific signal transduction pathways in airway epithelial cells. Using pharmacologic or genetic inhibition of either ERK or p38 MAP kinase pathway, we showed that ERK and p38 MAP kinases, but not JNK signaling, are essential for IL-1β- and TNF-α-induced *MUC5AC* gene expression. In addition, the activation of MSK1 and CREB is a crucial aspect of the intracellular mechanisms that mediate *MUC5AC* gene expression (Fig. 10), indicating that CREB activation is involved in the downstream signaling of MAP kinases and MSK1 for IL-1β- and TNF-α-induced *MUC5AC* gene expression. According to Fig. 8, IL-1β-activated CREB bound to a *cis*-acting element, CRE, in the *MUC5AC* promoter. Furthermore, this study also demonstrated that CRE in the *MUC5AC* promoter might play a role in these processes by binding to CREB. Further analysis of the signal pathways activated by various cytokines may yield deeper insights into the signal mechanism of *MUC5AC* gene expression.

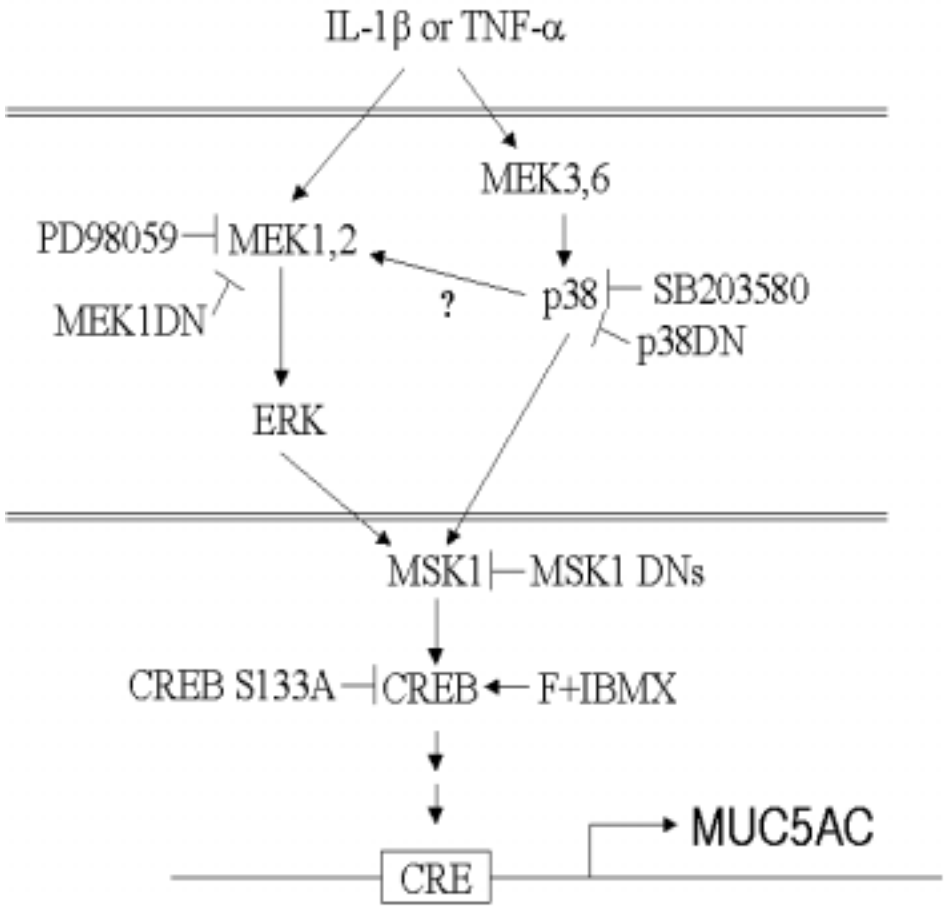

Fig. 10. Schematic diagram showing steps in the signaling pathway by which IL-1β or TNF-α up-regulated human *MUC5AC* gene transcription. As indicated, IL-1β or TNF-α activates a ERK and p38 MAP kinases-MSK1 pathway, which in turn leads to the activation of CREB-CRE and triggers *MUC5AC* gene expression in human airway epithelial cells.

REFERENCES

1. Yuta A, Ali M, Sabol M, Gaumond E, Baraniuk JN. Mucoglycoprotein hyper-secretion in allergic rhinitis and cystic fibrosis. Am J Physiol (Lung Cell Mol Physiol) 1997; 273: L1203-1207.
2. Kim SS, Kim KS, Lee JG, Park IY, Koo JS, Yoon JH. Levels of intracellular protein and messenger RNA of mucin and lysozyme in normal human nasal and polyp epithelium. Laryngoscope 2000; 110: 276-280.
3. Chung MH, Choi JY, Lee WS, Kim HN, Yoon JH. Compositional difference in middle ear effusion: mucous versus serous. Laryngoscope 2002; 112: 152-155.
4. Nadel JA. Role of epidermal growth factor receptor activation in regulating mucin synthesis. Respir Res 2001; 2: 85-89.
5. Spicer AP, Parry G, Patton S, Gendler SJ. Molecular cloning and analysis of the mouse homologue of the tumor-associated mucin, MUC1, reveals conservation of potential O-glycosylation sites, transmembrane, and cytoplasmic domains and a loss of minisatellite-like polymorphism. J Biol Chem 1991; 266: 15099-15109.
6. Gum JR Jr, Hicks JW, Toribara NW, Siddiki B, Kim YS. Molecular cloning of human intestinal mucin (MUC2) cDNA. Identification of the amino terminus and overall sequence similarity to prepro-von Willebrand factor. J Biol Chem 1994: 269: 2440-2446.

7. Van Klinken BJ, Van Dijken TC, Oussoren E, Buller HA, Dekker J, Einerhand AW. Molecular cloning of human MUC3 cDNA reveals a novel 59 amino acid tandem repeat region. Biochem Biophys Res Commun 1997: 238: 143-148.
8. Moniaux N, Nollet S, Porchet N, Degand P, Laine A, Aubert JP. Complete sequence of the human mucin MUC4: a putative cell membrane-associated mucin. Biochem J 1999; 338: 325-333.
9. Van de Bovenkamp JH, Hau CM, Strous GJ, Buller HA, Dekker J, Einerhand AW. Milecular cloning of human gastic mucin MUC5AC reveals conserved cysteine-rich D-domains and a putative leucine zipper motif. Biochem Biophys Res Commun 1998; 245: 853-859.
10. Keates AC, Nunes DP, Afdhal NH, Troxler RF, Offner GD. Molecular cloning of a major human gall bladder mucin: complete C-terminal sequence and genomic organization of MUC5B. Biochem J 1997; 324: 295-303.
11. Ho SB, Roberton AM, Shekels LL, Lyftogt CT, Niehans GA, Toribara NW. Expression cloning of gastic mucin complementary DNA and localization of mucin gene expression. Gastroenterology 1995; 109: 735-747.
12. Bobek LA, Tsai H, Biesbrock AR, Levine MJ. Molecular cloning, sequence, and specificity of expression of the gene encoding the low molecular weight human salivary mucin (MUC7). J Biol Chem 1993; 268: 20563-20569.
13. Shankar V, Pichan P, Eddy RL Jr, Tonk V, Nowak N, Sait SN, et al.

Chromosomal localization of a human mucin gene (MUC8) and cloning of the cDNA corresponding to the carboxy terminus. Am J Respir Cell Mol Biol 1997; 16: 232-241.

14. Lapensee L, Paquette Y, Bleau G. Allelic polymorphism and chromosomal localization of the human oviductin gene (MUC9). Fertil Steril 1997; 68: 702-708.
15. Melnick M, Chen H, Zhou Y, Jaskoll T. An alternatively spliced Muc10 glycoprotein ligand for putative L-selectin binding during mouse embryonic submandibular gland morphogenesis. Arch Oral Biol 2001; 46: 745-757.
16. Williams SJ, McGuckin MA, Gotley DC, Eyre HJ, Sutherland GR, Antalis TM. Two novel mucin genes down-regulated in colorectal cancer identified by differential display. Cancer Res 1999; 59: 4083-4089.
17. Williams SJ, Wreschner DH, Tran M, Eyre HJ, Sutherland GR, McGuckin MA. Muc13, a novel human cell surface mucin expressed by epithelial and hemopoietic cells. J Biol Chem 2001; 276: 18327-18336.
18. Pallesen LT, Berglund L, Rasmussen LK, Petersen TE, Rasmussen JT. Isolation and characterization of MUC15, a novel cell membrane-associated mucin. Eur J Biochem 2002; 269: 2755-2763.
19. Yin BW, and Lloyd KO. Molecular cloning of the CA125 ovarian cancer antigen: identification as a new mucin, MUC16. J Biol Chem 2001; 276: 27371-27375.
20. Gum JR Jr, Crawley SC, Hicks JW, Szymkowski DE, Kim YS. MUC17, a novel membrane-tethered mucin. Biochem Biophys Res Commun 2002; 291: 466-475.

21. Sers C, Kirsch K, Rothbacher U, Riethmuller G, Johnson JP. Genomic organization of the melanoma-associated glycoprotein MUC18: implications for the evolution of the immunoglobulin domains. Proc Natl Acad Sci USA 1993; 90: 8514-8518.

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

23. Thronton DJ, Howard M, Khan N, Sheehan JK. 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 1997; 272: 9561-9566.

24. Wickstrom C, Davies JR, Eriksen GV, Veerman EC, Carstedt I. 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 1998; 334: 685-693.

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

26. Gray T, Koo JS, Nettesheim P. Regulation of mucous differentiation and mucin gene expression in the tracheobronchial epithelium. Toxicology 2001; 160: 35-46.

27. Voynow JA, Young LR, Wang Y, Horger T, Rose MC, Fischer BM. Neutrophil elastase increases MUC5AC mRNA and protein expression in respiratory epithelial cells. Am J Physiol (Lung Cell Mol Physiol) 1999; 276:

L835-843.

28. Louahed J, Toda M, Jen J, Hamid Q, Renauld JC, Levitt RC, et al. Interleukin-9 upregulates mucus expression in the airways. Am J Respir Cell Mol Biol 2000; 22: 649-656.

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

30. Garrington TP, Johnson GL. Organization and regulation of mitogen-activated protein kinase signaling pathways. Curr Opin Cell Biol 1999; 11: 211-218.

31. Brunet A, Pouyssegur J. Identification of MAP kinase domains by redirecting stress signals into growth factor responses. Science 1996; 272: 1652-1655.

32. Bennett AM, Tonks NK. Regulation of distinct stages of skeletal muscle differentiation by mitogen-activated protein kinases. Science 1997; 278: 1288-1291.

33. Crawley JB, Rawlinson L, Lali FV, Paga TH, Saklatvala J, Foxwell BM. T cell proliferation in response to interleukins 2 and 7 requires p38MAP kinase activation. J Biol Chem 1997; 272: 15023-15027.

34. Foltz IN, Lee JC, Young PR, Schrader JW Hemopoietic growth factors with the exception of interleukin-4 activate the p38 mitogen-activated protein kinase pathway. J Biol Chem 1997; 272: 3296-3301.

35. Ichijo H, Nishida E, Irie K, Dijke PT, Saitoh M, Moriguchi T, and et al. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 1997; 275: 90-94.

36. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995; 270: 1326-1331.

37. Caivano M, Cohen P. Role of mitogen-activated protein kinase cascades in mediating lipopolysaccharide-stimulated induction of cyclooxygenase-2 and IL-1 beta in RAW264 macrophages. J Immunol 2000; 164: 3018-3025.

38. Arther JS, Cohen P. MSK1 is required for CREB phosphorylation in response to mitogens in mouse embryonic stem cells. FEBS Lett 2000; 482: 44-48.

39. Deak M, Clifton AD, Lucocq LM, Alessi DR. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. EMBO J 1998; 17: 4426-4441.

40. Wang B, Lim DJ, Han J, Kim YS, Basbaum CB, Li JD. Novel cytoplasmic proteins of nontypeable Haemophilus influenzae up-regulate human MUC5AC mucin transcription via a positive p38 mitogen-activated protein kinase pathway and a negative phosphoinositide 3-kinase-Akt pathway. J Biol Chem 2002; 277: 949-957.

41. Li JD, Feng W, Gallup M, Kim JH, Gum J, Kim Y, and et al. Activation of NF-kappaB via a Src-dependent Ras-MAPK-pp90rsk pathway is required for Pseudomonas aeruginosa-induced mucin overproduction in epithelial cells.

Proc Natl Acad Sci USA 1998; 95: 5718-5723.

42. Yoon JH, Kim KS, Kim SS, Lee JG, Park IY. Secretory differentiation of serially passaged normal human nasal epithelial cells by retinoic acid: expression of mucin and lysozyme. Ann Otol Rhinol Laryngol 2000; 109: 594-601.

43. Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, and et al. Proinflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem 1995; 270: 7420-7426.

44.Berrou E, Bryckaert M. Platelet-derived growth factor inhibits smooth muscle cell adhesion to fibronectin by ERK-dependent and ERK-independent pathways. J Biol Chem 2001; 276: 39303-39309.

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

46. Borchers MT, Carty MP, Leikauf GD. Regulation of human airway mucins by acrolein and inflammatory mediators. Am J Physiol (Lung cell Mol Physiol) 1999; 276: L549-555.

47. Ropert C, Almeida IC, Closel M, Travassos LR, Ferguson MA, Cohen P, and et al. Requirement of mitogen-activated protein kinases and I kappa B phosphorylation for induction of proinflammatory cytokines synthesis by macrophages indicates functional similarity of receptors triggered by glycosylphosphatidylinositol anchors from parasitic protozoa and bacterial lipopolysaccharide. J Immunol 2001; 166: 3423-3431.

48. Li D, Gallup M, Fan N, Szymkowski DE, Basbaum CB. Cloning of the amino-terminal and 5'-flanking region of the human MUC5AC mucin gene and transcriptional up-regulation by bacterial exoproducts. J Biol Chem 1998; 273: 6812-6820.

49. Gerritsen ME, Williams AJ, Neish AS, Moore S, Shi Y, Collins T. CREB-binding protein/p300 are transcriptional coactivators of p65. Proc Natl Acad Sci USA 1997; 94: 2927-2932.

50. Perrais M, Pigny P, Copin MC, Aupert JP, Van Seuningen I. Induction of MUC2 and MUC5AC mucins by factors of the epidermal growth factor (EGF) family is mediated by EGF receptor/Ras/Raf/extracellular signal-regulated kinase cascade and Sp1. J Biol Chem 2002; 277: 32258-32227.

51. Van Seuningen I, Pigny P, Perrais M, Porchet N, Aubert, JP. Transcriptional regulation of the 11p15 mucin genes. Towards new biological tools in human therapy, in inflammatory diseases and cancer? Front Biosci 2001; 6: D1216-1234.

52. Perrais M, Pigny P, Buisine MP, Porchet N, Aubert JP, Van Seuningen I. Aberrant expression of human mucin gene MUC5B in gastric carcinoma and cancer cells. Identification and regulation of a distal promoter. J Biol Chem 2001; 276: 15386-15396.

53. Chung CC, Huang D, Chen Y, Short S, Short ML, Zhang Z, and et al. Identification of a silencer module which selectively represses cyclic AMP-responsive element-dependent gene expression. Mol Cell Biol 1995; 15: 6139-6149.

국문요약

정상 호흡기 상피 세포에서 IL-1β와 TNF-α에 의한 *MUC5AC* 유전자발현에서 ERK/p38 MAP kinase-MSK1-CREB 신호전달체계 규명

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송 경 섭

점액과분비는 호흡기에서 염증을 수반하는 질환에서 보통 관찰되어진다. 인간 정상 호흡기의 goblet 세포에서 매우 많이 발현되는 mucin이 MUC5AC이기 때문에 MUC5AC는 호흡기에서 매우 중요한 mucin으로 알려져 있다. 더욱이 이러한 MUC5AC의 발현은 다양한 염증 매개체에 의해 조절되어진다고 알려져 있다. 그러나 정상 코 상피 세포에서 중요한 염증 매개체로 알려진 IL-1β나 TNF-

α에 의한 MUC5AC 발현 신호전달은 아직까지 정확히 밝혀지지 않았다. 본 연구에서는 IL-1β나 TNF-α가 어떠한 기전으로 MUC5AC 발현을 조절하는지 알아보기 위하여 생화학적인 방법과 유전학적인 방법을 사용하여 조사하였다. IL-1β나 TNF-α에 의해 MAP kinase 중에서 ERK와 p38 MAP kinase 신호 전달 단백질이 관여함을 알았고 이러한 단백질을 억제하였을 때 MUC5AC 발현이 억제 되는 것으로 관찰되었다. 이러한 결과는 ERK와 p38 MAP kinase가 IL-1β나 TNF-α에 의한 MUC5AC 발현신호전달에 필수적임을 알았다. 또한 ERK와 p38 MAP kinase의 downstream에 MSK1과 CREB이 관여함을 유전학적인 방법으로 증명하였고, IL-1β나 TNF-α에 의해 활성화된 CREB은 *MUC5AC* promoter에 존재하는 CRE에 결합하는 것을 관찰하였다. 더욱이 *MUC5AC* promoter의 CRE (-878)에 의해 전사가 일어남을 관찰하였다. 이러한 결과들에 의해서 IL-1β나 TNF-α에 의해 *MUC5AC* 발현 기전은 순차적으로 ERK/p38 MAP kinase-MSK1-CREB-CRE 기전임을 증명하였다. 이러한 연구는 IL-1β나 TNF-α에 의한 MUC5AC 발현신호전달을 증명함으로써 염증반응 동안에 mucin 과분비를 이해하는데 중요한 자료를 제시한다.

핵심되는 말: CRE, CREB, ERK, MSK1, *MUC5AC*, p38.