THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 37, Issue of September 12, pp. 34890–34896, 2003
Printed in U.S.A.

Induction of *MUC8* Gene Expression by Interleukin-1β Is Mediated by a Sequential ERK MAPK/RSK1/CREB Cascade Pathway in Human Airway Epithelial Cells*

Received for publication, April 14, 2003, and in revised form, June 16, 2003
Published, JBC Papers in Press, July 3, 2003, DOI 10.1074/jbc.M303911200

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Mucins are the major components of the mucus layer that covers and protects the respiratory, digestive, and reproductive tracts. Our previous studies showed that *MUC8* gene expression was overexpressed in *in vivo* polyp epithelium in chronic sinusitis and was also increased by treatment with inflammatory mediators in an *in vitro* culture condition. However, the mechanisms by which the inflammatory mediators-induced *MUC8* gene expression in normal nasal epithelial cells evolved remain unclear. We examined the mechanism by which the important proinflammatory mediator, interleukin (IL)-1β, increases *MUC8* gene expression levels. We found that pharmacologic and genetic inhibition of ERK MAPK pathway abolished IL-1β-induced *MUC8* gene expression in normal human nasal epithelial cells. Moreover, the overexpression of wide-type or of the dominant-negative mutant of p90 ribosomal S6 protein kinase 1 (RSK1) enhanced or suppressed, respectively, IL-1β-induced *MUC8* gene expression. RSK1 was found to directly phosphorylate cAMP-response element-binding protein (CREB), and this event led to the stimulation of subsequent CRE-mediated gene transcription. In conclusion, IL-1β was found to induce *MUC8* gene expression via a sequential ERK/RSK1/CREB pathway in human airway epithelial cells.

Mucins are highly glycosylated, high molecular mass glycoproteins and are major components of the mucus produced by the epithelia of the respiratory, gastrointestinal, and reproductive tracts. They are responsible for the viscoelastic properties of secreted mucus and provide lubrication and protection for mucus membranes (1). In the airway, virtually all forms of airway inflammation are associated with the overproduction of mucus, which can lead to airway obstruction (2).

Eighteen types of mucin genes have been discovered to date: *MUC1* to *MUC4*, *MUC5AC*, *MUC5B*, *MUC6*, *MUC7*, *MUC8* (2), *MUC9* (3), *MUC10* (4), *MUC11*, *MUC12* (5), *MUC13* (6), *MUC15* (7), *MUC16* (8), *MUC17* (9), and *MUC18* (10). Of these, MUC5AC and MUC5B are known to be major gel-foaming mucins secreted in the human airway. Accordingly, most studies on mucin genes have been focused on these two mucins. However, although MUC5AC is known to be expressed by most surface goblet cells, we found that only a portion of the goblet cells (11) expressed *MUC5AC* mRNA. This suggests that other mucin genes in addition to *MUC5AC* might be important for mucus hypersecretion. We have previously investigated the expressions of other *mucin* genes using various inflammatory tissues and cell lysates. Interestingly, *in vivo*, we found that *MUC8* mRNA levels are clearly up-regulated in the polyp epithelium, which is invariably stimulated by inflammatory mediators (12). In addition, *in vitro*, interleukin-1β (IL-1β),[1] tumor necrosis factor-α, and a mixture of inflammatory mediators were found to up-regulate *MUC8* mRNA and to down-regulate *MUC5AC* mRNA (13, 14). These results showed that *MUC8* mRNA is increased both *in vivo* and *in vitro* during inflammatory conditions. However, the mechanisms of *MUC8* gene expression during inflammation in normal airway epithelial cells and the signal molecules involved have not been elucidated.

Mitogen-activated protein kinases (MAPKs) are ubiquitous kinases and are involved in signal transduction in eukaryotic organisms. This family of kinases is characterized by their activation by MAPKs through the dual phosphorylation of Thr and Tyr residues in their activation loop. The MAPK family includes extracellular signal-regulated kinases (ERK), which are activated in response to growth factors, via the Ras proto-oncogene. Moreover, c-Jun N-terminal kinase (JNK) and p38 MAPK constitute two other families, collectively known as stress-activated protein kinases (SAPK), because they are induced by UV radiation, heat-shock, oxidative stress, or tumor necrosis factor-α. The stimulation of ERK initiates a cascade of

* This study was supported by the 2002 Good Health R&D project, Ministry of Health and Welfare, South Korea (Grant 02-PJ1-PG3-20908-0005). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: IL-1β, interleukin 1β; MUC, mucin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK1, MAPK/ERK kinase 1; SAPK, stress-activated protein kinase; MSK1, mitogen- and stress-activated protein kinase 1; RSK1, p90 ribosomal S6 protein kinase 1; CRE, cAMP-response element; CREB, CRE-binding protein; EMSA, electrophoretic mobility shift analysis; W/T, wide-type; DN, dominant-negative; JNK, c-Jun NH_2-terminal kinase; NHNE, normal human nasal epithelial; FBS, fetal bovine serum; PBS, phosphate-buffered saline; FAM, carboxyfluorescein; TAMRA, carboxytetramethylrhodamine; DTT, dithiothreitol; IBMX, isobutylmethylxanthine.

activating events, including the phosphorylation of p90 ribosomal S6 protein kinase 1 (RSK1), and its translocation to the nucleus, where RSK1 phosphorylates nuclear substrates (15). Moreover, the phosphorylation of mitogen- and stress-activated protein kinase (MSK), which localized in the nuclei (16), could lead to the phosphorylation and activation several transcription factors like cAMP-response element-binding protein (CREB) and activating transcription factor 1 (ATF1) (17).

We examined the mechanism by which the important proinflammatory mediator, IL-1β, increases *MUC8* gene expression levels. Here we show that ERK MAPK is essential for IL-1β-induced *MUC8* gene expression in normal human nasal epithelial (NHNE) cells. We also show that RSK1 mediates the IL-1β-induced phosphorylation of CREB and CRE-mediated transcription. Molecular cloning of the *MUC8* promoter regulated by various stimuli may yield a deeper insight into cellular function.

EXPERIMENTAL PROCEDURES

Materials—PD98059, SB203580, and anti-α-tubulin antibody were purchased from Calbiochem (San Diego, CA). Anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) antibody, anti-phospho-p38 MAPK (Thr^{180}/Tyr^{182}) antibody, anti-phospho-SAPK/JNK MAPK (Thr^{183}/Tyr^{185}) antibody, anti-phospho-RSK1 (Ser^{380}) antibody, and anti-phospho-CREB (Ser^{133}) antibody were purchased from Cell Signaling (Beverly, MA). cDNA construct encoding dominant-negative Raf1 construct was kindly provided by Dr. J. H. Kim (University of Korea, Seoul, Korea).

Cell Cultures—The culture system used for the normal human nasal epithelial (NHNE) cells has been previously reported (13). The human lung mucoepidermoid carcinoma cell line, NCI-H292, was purchased from the American Type Culture Collection (CRL-1848, Manassas, VA) and was cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin-streptomycin at 37 °C in a 5% CO_2 humidified chamber. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline (PBS) and re-cultured in RPMI 1640 with 0.2% FBS.

Real-time Quantitative PCR—Primers and probes were designed using PerkinElmer Life Sciences Primer Express® software, purchased from PE Biosystems. Commercial reagents (TaqMan PCR Universal PCR Master Mix, PE Biosystems) and conditions were applied according to the manufacturer's protocol. One microgram of cDNA (reverse transcription mixture), oligonucleotides at a final concentration of 800 nM of primers, and 200 nM TaqMan hybridization probe were used in a 25-μl volume. The probe of real-time PCR was labeled with carboxyfluorescein (FAM) at the 5′-end and with the quencher carboxytetramethylrhodamine (TAMRA) at the 3′-end. The following primers and TaqMan probes were used: *MUC8*, forward (5′-GACCTGCCCCCATGGAC-3′) and reverse (5′-CAGGAGTTCGAGACCAGCCT-3′) and TaqMan probe (6FAM-CCACCTCCGAGCCCGTCACTGAG-TAMRA). *β_2M*, forward (5′-CGCTCCGTGGCCTTAGC-3′) and reverse (5′-GAGTACGCTGGATAGCCTCCA-3′) and TaqMan probe (6FAM-TGCTCGCGCTACTCTCTCTTTCTGGC-TAMRA). Real-time reverse transcription-PCR was performed on a PE Biosystems ABI PRISM® 7700 Sequence Detection System (Foster City, CA). The thermocycler (ABI PRISM® 7700 Sequence Detection System) parameters were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were performed in triplicate. Relative quantity of *MUC8* mRNA was obtained using a comparative cycle of threshold method, and results were normalized against *β_2M* as an endogenous control.

Western Blot Analysis—NCI-H292 cells were grown to confluence in 6-well plates. After 15 or 45 min of treatment with IL-1β, the cells were lysed with 2× lysis buffer (250 mM Tris-Cl (pH 6.5), 2% SDS, 4% β-mercaptoethanol, 0.02% bromophenol blue, 10% glycerol). Equal amounts of whole cell lysates were resolved by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were blocked with 5% skim milk in Tris-buffered saline (50 mM Tris-Cl (pH 7.5), 150 mM NaCl) for 2 h at room temperature. This membrane was then incubated overnight with primary antibody in TTBS (0.5% Tween 20 in Tris-buffered saline). 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 using the ECL system (Amersham Biosciences).

Preparation of an Inducible Dominant-negative Mutant Stable Cell Line—Plasmid encoding the kinase-deficient MEK1 mutant (*pcDNA5-MEK1DN*) was cut with *Bam*H1 (Promega, Madison, WI) and ligated with pBluescript (Stratagene, La Jolla, CA). This clone was cut with *Hin*dIII (Promega), filled in with Klenow, re-cut with *Sac*II (Promega), and then ligated to *pTRE* vector (Clontech, Palo Alto, CA). Plasmid encoding kinase-inactive p38 mutant (*pcDNA3-p38AGF*) was cut with *Bam*H1, filled in with Klenow, re-cut with *Xba*I (Promega), and then ligated to *pTRE* vector. NCI-H292 cells were then 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), 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.

Immunoprecipitation—One microgram of polyclonal anti-phospho CREB antibody was incubated at 4 °C overnight with 400 μg of cell extract prepared using lysis buffer (20 mM Tris-Cl (pH 7.9), 1% Triton X-100, 1 mM Na_3VO_4, 137 mM NaCl, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM EGTA, 10 mM NaF, 1 mM $Na_4P_2O_7$, 5 mM Na_2EDTA, 10% glycerol, 1 mM β-glycerophosphate, 0.1 g/ml *p*-nitrophenyl phosphate, 0.2 mM phenylmethylsulfonyl fluoride). Forty microliters of a 1:1 suspension of protein A-Sepharose beads was added to the cell lysates and incubated for 2 h at 4 °C, with gentle rotation. The beads were pelleted and washed extensively with cell lysis buffer. Bound proteins were dissociated by boiling the samples in PAGE sample buffer, and whole samples were separated on SDS-PAGE gel.

In Vitro RSK1 Assay—For serum deprivation, confluent cells were washed twice with PBS and recultured in RPMI 1640 containing 0.2% FBS. Cells were treated with IL-1β for 30 min, harvested, and lysed in lysis buffer. 300 μg of the protein so obtained was incubated with anti-phospho-RSK1 (Ser^{380}) antibody overnight at 4 °C. Immunocomplexes were added to 40 μl of protein A-Sepharose beads. Samples were washed three times in lysis buffer, and kinase reactions were carried out for 1 h at 30 °C in 20 μl of kinase buffer (20 mM HEPES (pH 7.2), 5 mM $MnCl_2$, 200 μM sodium orthovanadate, 5 μg of acid-treated enolase, 10 μM ATP, 5 μCi of [γ-^{32}P]ATP) and 5 μg of bacterially expressed glutathione *S*-transferase (GST)-CREB as a substrate. Reactions were stopped by adding SDS-PAGE sample buffer and analyzed by SDS-PAGE followed by autoradiography.

Electrophoretic Mobility Shift Analysis—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 phenylmethylsulfonyl fluoride, 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 phenylmethylsulfonyl fluoride, 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 the consensus CRE sequences (5′-AGAGATTGCCTGACGTCAGAGAGCTAG-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 0.5× 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 °C.

Transient Transfection and Luciferase Assay—Luciferase reporter construct (*pCRE-luc*) and plasmid expressing mutant CREB (*pCREB S133A*, with serine residue 133 replaced by alanine) vector, were transiently transfected using a FuGENE6 transfection reagent (Roche Applied Science), according to the manufacturer's instructions. Cells were incubated for 48 h, harvested, and assayed for luciferase activity, using a luciferase assay system (Promega), according to the manufacturer's instructions. β-Galactosidase activity was also assayed to standardize the transfection efficiencies.

RESULTS

Effects of ERK MAPK on IL-1β-induced MUC8 Gene Expression—Previously, ERK and p38 MAPKs had shown maximum activation after 15-min treatment with IL-1β, this effect decreased at 45 min in NHNE cells. No change was detected in

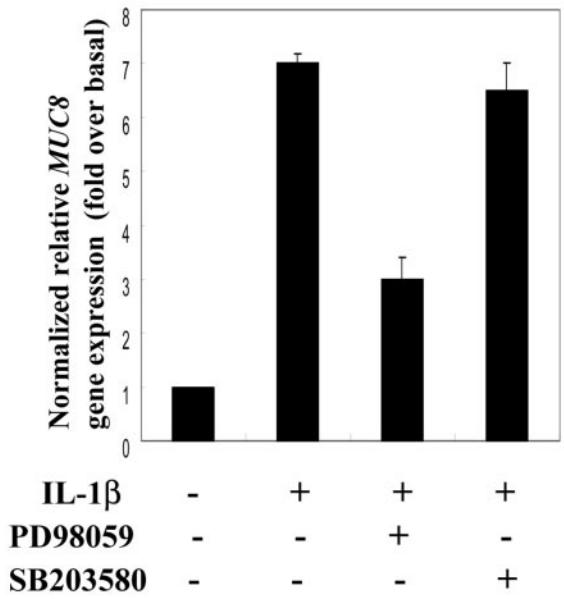

FIG. 1. **Effect of ERK or p38 MAPK on *MUC8* gene expression in NHNE cells.** Confluent cells were pretreated for 1 h with 20 μM PD98059 or 20 μM SB203580 and then stimulated for 24 h with IL-1β prior to the collection of total RNA for the real-time quantitative PCR of *MUC8*. The figures shown are representative of three independent experiments.

the activation of JNK (18). To investigate the possible involvement of ERK or p38 MAPK pathway in IL-1β-induced *MUC8* gene expression, we performed real-time PCR after pretreatment with 20 μM PD98059 or 20 μM SB203580 for 1 h. Real-time quantitative PCR showed that pretreatment with PD98059 for 1 h inhibited *MUC8* gene expression (Fig. 1). However, pretreatment with SB203580 did not affect *MUC8* gene expression (Fig. 1). These results indicate that the activation of ERK MAPK, but not of p38 MAPK, appeared to be closely related to IL-1β-induced *MUC8* gene expression.

When the same experiment (Fig. 1) was performed using NCI-H292 cells, a human lung mucoepidermoid carcinoma cell line, we obtained results that were similar to those of normal cells (data not shown). To further confirm the significance of ERK or p38 kinase upon the cellular level of *MUC8* gene expression, we generated cells stably expressing dominant-negative (DN) mutant MEK1 or p38 under control of the Tet-off system. After removing doxycycline to induce MEK1DN, cells were stimulated with IL-1β for 15 min, and IL-1β-induced phosphorylation of ERK significantly decreased (Fig. 2*A*). However, no change in ERK expression was observed. Real-time quantitative PCR showed a significant decrease in *MUC8* gene expression after 24 h (Fig. 2*A*). In a similar way, we investigated the role of p38 MAPK on IL-1β-induced *MUC8* gene expression using p38DN. An *in vitro* kinase assay showed that the activation of p38 MAPK in this mutant stable cell lines was reduced in the presence of IL-1β (Fig. 2*B*). However, the IL-1β-induced *MUC8* gene expression was not affected by p38DN induction. These results showed that ERK MAPK, but not p38 MAPK, was essential for IL-1β-induced *MUC8* gene expression in NCI-H292 cells. To examine whether the sequential Ras/Raf/MEK1/ERK pathway plays a role in IL-1β-induced *MUC8* gene expression, we transiently transfected DNA with constructs encoding RasDN (Ras N17) or Raf1DN (craf1, a kinase-defective form of Raf1). The transient overexpressions of Ras or Raf1 DN did not affect the IL-1β-activated phosphorylation of ERK MAPK (Fig. 2*C*), and the IL-1β-induced *MUC8* gene expression (Fig. 2*D*). ECV304 cells were used as a positive control for these dominant-negative vectors (19). These results show that the activation of ERK MAPK via MEK1 by IL-1β might occur via a Ras-independent pathway to induce *MUC8* gene expression in airway epithelial cells.

Effects of RSK1 on IL-1β-induced MUC8 Gene Expression—To determine which molecules are involved in the downstream signaling of ERK MAPK in IL-1β-induced *MUC8* gene expression, we investigated RSK1 and MSK1. RSK1 has been reported to be activated by ERK MAPK (20–22). The phosphorylation of RSK1 by IL-1β peaked at 30 min and then decreased at 60 min after IL-1β stimulation (Fig. 3*A*). Pretreatment with 20 μM PD98059 inhibited IL-1β-induced RSK1 phosphorylation (Fig. 3*B*), indicating that RSK1 is regulated by ERK MAPK. These results showed that RSK1 acts as a downstream signaling mediator of ERK MAPK. To determine whether RSK1 plays an important role in IL-1β-induced *MUC8* gene expression, an RSK1 mutant study was performed. RSK1 is currently a candidate for the mediation of cytokine-induced CREB phosphorylation at Ser^{133} (20, 23, 24). Overexpression of wide-type (W/T) RSK1 increased IL-1β-induced CREB phosphorylation. However, the overexpression of RSK1DN (D205N) suppressed the IL-1β-induced CREB phosphorylation. Consistently, IL-1β-induced *MUC8* gene expression was increased by overexpression of W/T RSK1, whereas the IL-1β-induced *MUC8* gene expression was significantly suppressed by RSK1DN (D205N) (Fig. 3*C*). These results show that RSK1 is required for IL-1β-induced *MUC8* gene expression.

Effects of CREB on IL-1β-induced MUC8 Gene Expression—We examined whether IL-1β-activated RSK1 specifically binds to CREB in human airway cells. Cell extracts obtained after stimulation with IL-1β were immunoprecipitated with anti-phospho CREB antibody and then blotted with an anti-phospho RSK1 antibody. As shown in Fig. 4*A*, protein complex formation was observed between RSK1 and CREB. The control IgG was used as an immunoprecipitating antibody. In addition, a transient phosphorylation of CREB was observed upon the stimulation with IL-1β, reaching the maximum peak at 30 min. These results suggest that IL-1β-induced RSK1 leads to the phosphorylation of CREB in NCI-H292 cells. To confirm whether IL-1β-induced phospho-RSK1 is enzymatically active, we performed an immunocomplex *in vitro* kinase assay using bacterially expressed GST-CREB as a substrate. As shown in Fig. 4*B*, IL-1β-induced RSK1 activity remarkably increased in cells transfected with W/T RSK1, whereas the overexpression of RSK1 DN diminished IL-1β-induced RSK1 activity.

To determine whether CREB plays a role in *MUC8* gene expression, we used both forskolin (an activator of adenylate cyclase) and 3-isobutyl-1-methylxanthine (IBMX, an inhibitor of AMP phosphodiesterase). The transient phosphorylation of CREB was observed after stimulation with both forskolin and IBMX, and this reached a maximum peak at 10 min (18). The cAMP pathway-induced CREB phosphorylation increased *MUC8* gene expression (Fig. 4*C*). Furthermore, IL-1β-induced *MUC8* gene expression was significantly suppressed in cells transfected with plasmid encoding CREBDN (S133A) (Fig. 4*D*). These findings suggest that the activation of CREB is essential for IL-1β-induced *MUC8* gene expression via ERK MAPK and RSK1.

CREB Activates CRE-mediated Gene Transcription in Response to IL-1β—To determine the DNA binding activity of IL-1β-activated CREB, we performed EMSA using nuclear extracts from NCI-H292 cells after treatment with IL-1β for 1 h. As shown in Fig. 5*A*, the activity of consensus CRE oligonucleotide remarkably increased in response to IL-1β. To identify specific CRE binding complex, competition and supershift analysis were performed using a 50-fold excess of nonradiolabeled (cold) CRE oligonucleotide and an anti-phospho-CREB anti-

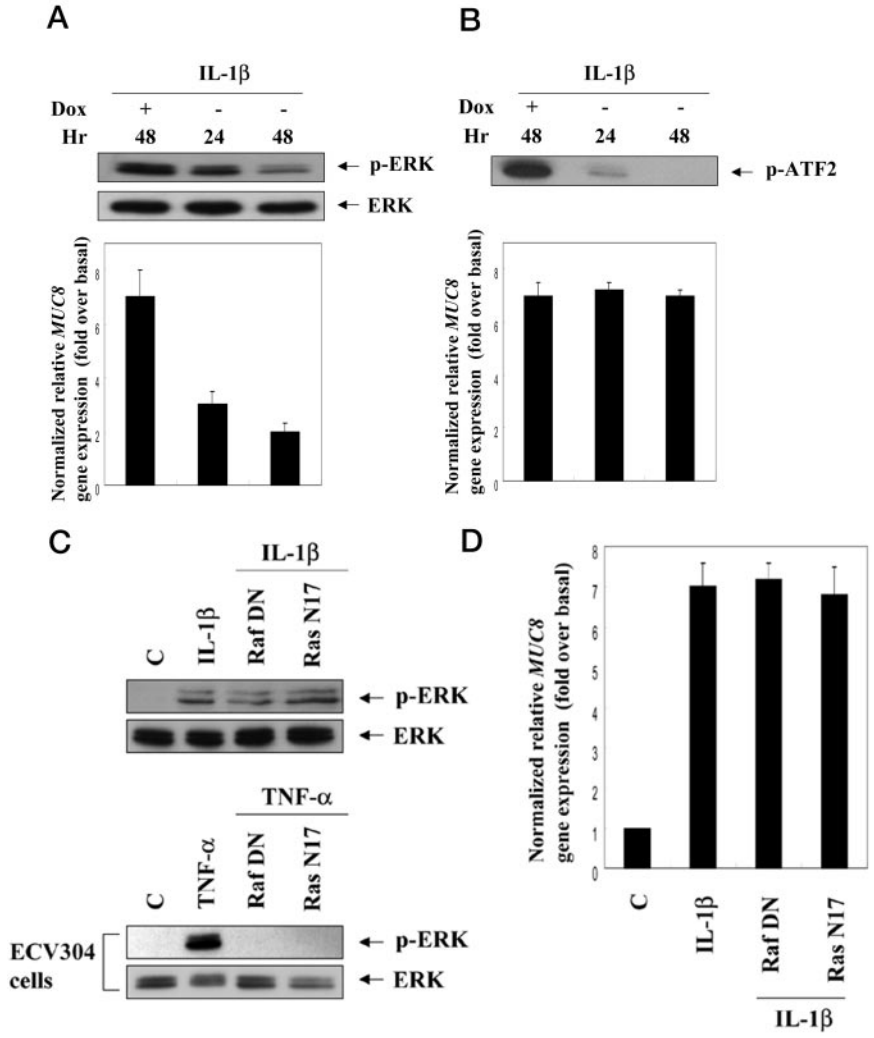

FIG. 2. ***MUC8* gene expression in MEK1 or p38 dominant-negative mutant stable cell lines.** Confluent, quiescent MEK1 (*A*) and p38 (*B*) dominant-negative mutant stable cells were preincubated for the indicated times to induce dominant-negative mutant protein by removing doxycycline and then stimulating for 15 min with IL-1β prior to Western blotting and for 24 h with IL-1β prior to real-time quantitative PCR. For the other experiments, cells were transiently transfected with cDNA constructs encoding dominant-negative Raf1 or Ras and stimulated with IL-1β for 15 min prior to Western blot analysis with phospho ERK antibody (*C*) and for 24 h prior to real-time quantitative PCR (*D*). *C*, control, ECV304 cells were employed as a positive control. The figures shown are representative of three independent experiments.

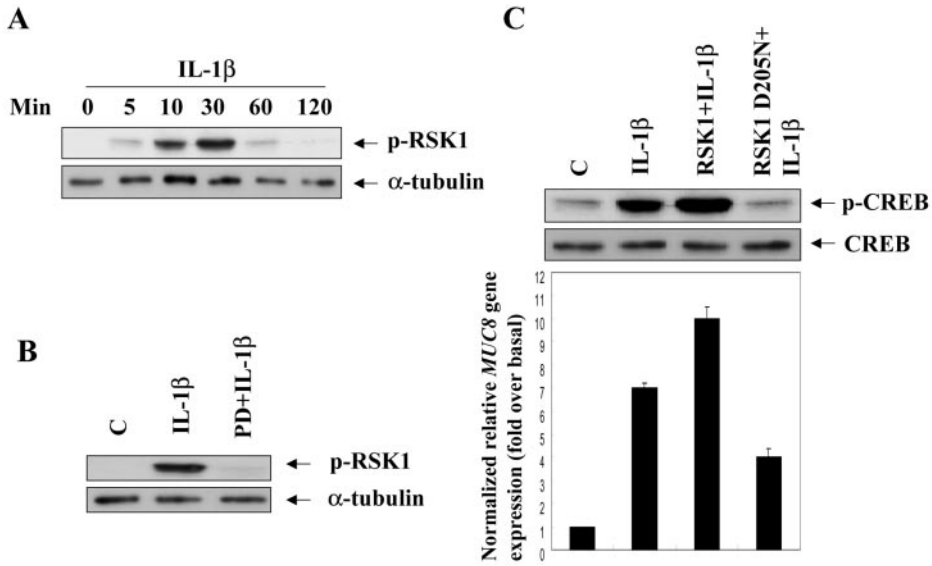

FIG. 3. **Effect of RSK1 on IL-1β-induced *MUC8* gene expression.** *A*, confluent, quiescent cells were stimulated for the indicated times with IL-1β, and then total proteins were collected for Western blot analysis using phospho-RSK1 antibody. *B*, for the other experiments, the cells were pretreated for 1 h with 20 μM PD98059 and then stimulated with IL-1β for 30 min prior to Western blot analysis. Cells were transiently transfected with wide-type or mutant RSK1 D205N construct and stimulated with IL-1β for 30 min prior to Western blot analysis (*C*, *upper panel*) and for 24 h prior to real-time quantitative PCR (*C*, *lower panel*). *C*, control. The figures shown are representative of three independent experiments.

body, respectively. Specific band was found to be selectively inhibited by the specific CRE competitor and to be supershifted by anti-phospho-CREB antibody. These results indicate that activated CREB binds to a *cis*-acting element, which we refer to as CRE. Next, we assayed the gene expression of the *pCRE-luc* construct to determine whether IL-1β exerts a stimulatory effect on the activation of CREB and on the subsequent CRE-mediated gene transcription. To investigate the role of CREB phosphorylation on CRE-mediated gene transcription, cells were transfected transiently with *pCRE-luc* vector only or both *pCRE-luc* vector and plasmid encoding CREB DN (S133A). Treatment of transfected NCI-H292 cells with IL-1β resulted in increased CRE-mediated gene transcription in a time-dependent manner (Fig. 5*B*). The expression of CREB containing a

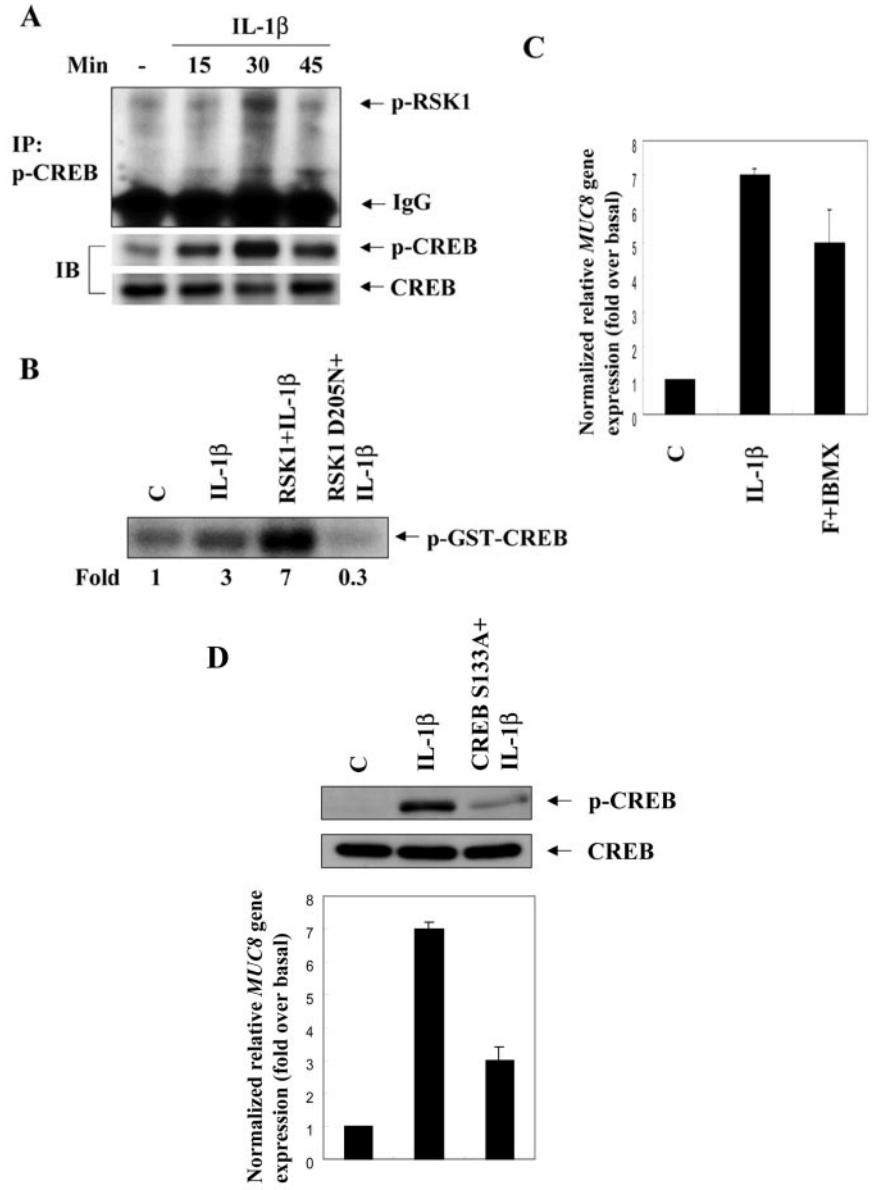

FIG. 4. **Effect of CREB on IL-1β-induced *MUC8* gene expression.** Confluent, quiescent cells were stimulated for the indicated times with IL-1β. *A*, total cell lysates were then immunoprecipitated with anti-phospho CREB antibody and blotted with anti-phospho-RSK1 antibody. *B*, an *in vitro* RSK1 kinase assay was performed using bacterially expressed GST-CREB as an exogenous substrate. Levels of phosphorylated CREB were visualized by autoradiography. *C*, cells were stimulated for 24 h with IL-1β or both 20 μM forskolin and 5 μM IBMX, and the total RNA were then subjected to real-time quantitative PCR. Cells were transiently transfected with mutant CREB (*pCREB S133A*) constructs and stimulated with IL-1β for 30 min prior to Western blot analysis (*D*, *upper panel*) and for 24 h prior to real-time quantitative PCR (*D*, *lower panel*). *IP*, immunoprecipitation; *IB*, immunoblotting; *C*, control. The figures shown are representative of three independent experiments.

mutation of the critical regulatory Ser^{133} residue was found to significantly inhibit the luciferase activity induced by IL-1β. Mock transfection, used as a negative control, showed no significant induction of CRE-mediated reporter transcription. These results suggest that IL-1β-activated CREB triggers CRE-mediated transcription by binding to CRE.

DISCUSSION

Mucociliary clearance is an important function of the airway epithelium. Human beings inhale noxious gases, air pollutants, bacteria, and viruses through the nose, and these are usually trapped by mucus and removed by ciliary beating toward the nasopharynx. Increased mucus secretion during inflammation may represent a defensive mechanism, and inflammatory mediators increase the ciliary beating of respiratory epithelial cells to promote mucociliary clearance (25–27).

The molecular mechanism by which *MUC8* gene expression is up-regulated by IL-1β remains poorly understood. In the present study, we investigated the mechanisms by which *MUC8* gene expression is up-regulated by IL-1β in normal human nasal epithelial cells. Our results show that only the activation of ERK MAPK was required for IL-1β-induced *MUC8* gene expression, although several reports have concluded that more than one MAPK might be necessary for the signal transduction of various inflammatory mediators (28–30). Moreover, the ERK MAPK cascade is known to be activated by G-protein-coupled receptor via: (i) EGFR transactivation (Ras-dependent); (ii) a protein kinase C-dependent, but epidermal growth factor receptor- and Ras-independent, pathway; or (iii) a Ras-independent and cAMP/protein kinase A-mediated pathway (31). Of these pathways, the activation of ERK MAPK is known to be mainly mediated by Ras (32). In this study, we investigated whether IL-1β-induced activation of ERK MAPK is Ras-dependent or -independent. The overexpression of Raf1 DN (craf1) and Ras DN (RasN17) mutants did not affect the IL-1β-induced phosphorylation of ERK MAPK and *MUC8* gene expression (Fig. 2, *C* and *D*). These results show that activation of ERK MAPK by MEK1, as induced by IL-1β, might occur via a Ras/Raf-independent pathway to induce *MUC8* gene expression in human airway epithelial cells. Further work is necessary to explore these pathways.

To date, the signal molecules involved in the downstream signaling of ERK MAPK, for IL-1β-induced *MUC8* gene expression, have not been yet identified. The role of RSK1 and CREB in the downstream signaling of ERK MAPK to induce *MUC8*

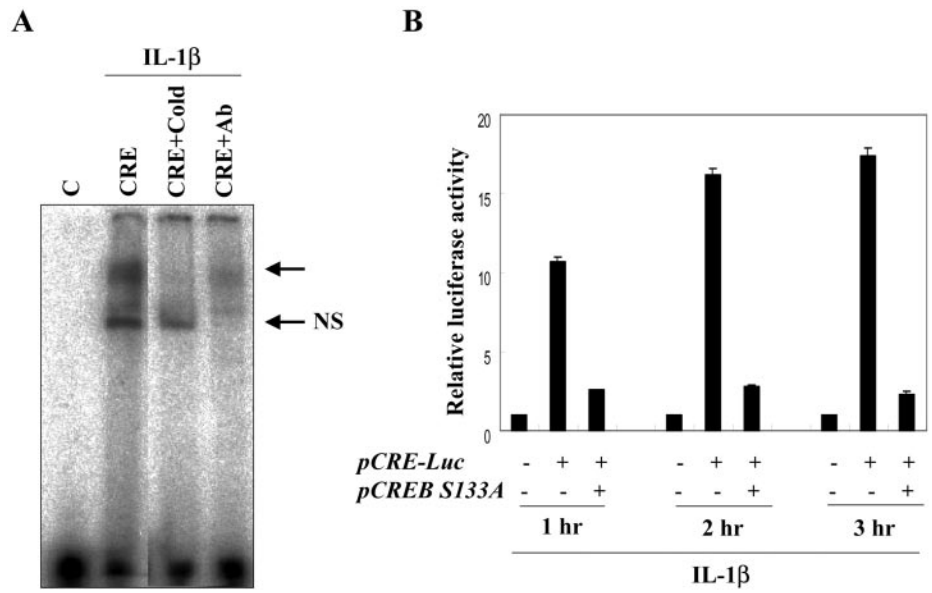

FIG. 5. **IL-1β-induced activation of CRE-mediated gene transcription via the *cis*-acting regulatory CRE motif.** Confluent, quiescent cells were stimulated for 1 h with IL-1β. Nuclear proteins were incubated with CRE, a 50-fold excess of cold probe, or anti-phospho CREB antibody before EMSA. *A*, the labeled nuclear proteins were separated by electrophoresis on 5% polyacrylamide gels, and the gels were dried and autoradiographed at −70 °C overnight. *C*, control; *Ab*, antibody; *NS*, nonspecific. *B*, when the cells were 70% confluent, *pCRE-luc*, or both *pCRE-luc* and CREB DN (*pCREB S133A*) plasmid, and empty parental control vector (mock) as a control, were transiently transfected into the NCI-H292 cells. Confluent cells were then stimulated with IL-1β for the indicated times, and the luciferase activity of the reporter plasmid was measured. Luciferase activities were determined after correcting for transfection efficiency *versus* the β-galactosidase activity of the cell lysates. The values shown are means ± S.D. of experiments performed in triplicate.

gene expression is a major finding of the present study. The substrates of ERK MAPK are known to be the MSK and RSK family members (33). Our results show that RSK1 might be essential for IL-1β-induced *MUC8* gene expression. In addition, the activation of MSK1 appears to be closely related to IL-1β-induced *MUC5AC* gene expression by IL-1β in human airway epithelial cells (18). Differences in the activations of these kinases by IL-1β may be responsible for differences in target genes. RSK1 phosphorylates several transcription factors, including CREB (34), c-Fos (35), CCAAT/enhancer binding protein (36), nuclear factor-κB (21), and the estrogen receptor (37), and interacts with transcriptional coactivator CREB-binding protein (also known as p300) (38). Many studies have shown that RSK1 phosphorylates Ser^{133} of CREB (16, 39–41). Although CREB activation by RSK1 has been established by previous studies in other cells, and CREB is a potent regulator of mucin (*MUC2*, *MUC5AC*, *MUC5B*, and *MUC6*) gene expression in the p15 arm of chromosome 11 (11p15) (17), its role remains unclear in airway epithelial cells. In addition, little is known about the involvement of CREB in *MUC8* gene expression. In the present study, the activation of CREB was found, at least in part, essential for IL-1β-induced *MUC8* gene expression via ERK MAPK and RSK1. Interestingly, *MUC8* gene expression was inhibited in CREB DN (S133A)-transfected cells treated with IL-1β. In addition, treatment with both forskolin and IBMX activated the phosphorylation of CREB and increased *MUC8* gene expression (Fig. 4, *C* and *D*). These results suggest that CREB might be a transcription factor for IL-1β-induced *MUC8* gene expression. However, increased *MUC8* expression induced by both forskolin and IBMX was less than that induced by IL-1β, indicating that activation by CREB alone is insufficient for IL-1β-induced *MUC8* gene expression. These results suggest that a transcription factor, other than CREB, may be required for IL-1β-induced *MUC8* gene expression.

We examined whether IL-1β-induced *MUC8* gene expression in human airway epithelial cells is a CRE-mediated transcription. We found that the IL-1β-induced phosphorylation of CREB had the DNA binding activity to CRE (Fig. 5*A*). Moreover, IL-1β-induced CRE activation increased in a time-dependent manner, whereas the overexpression of CREB DN mutant led to a ~90% decrease in the response of the CRE minimal promoter to IL-1β (Fig. 5*B*). These results showed that the activation of the *cis*-element, CRE, appeared closely related to IL-1β-induced *MUC8* gene expression in human airway epithelial cells. These results indicate that the *MUC8* promoter might have CRE site(s) and the CRE might be an important transcription factor of the *MUC8* promoter, like the *mucins* of 11p15 chromosome (17). However, unfortunately, the promoter and cDNA sequences of the *MUC8* gene have not yet been fully identified. Thus, further studies upon the *MUC8* promoter seem warranted.

In summary, IL-1β was found to induce *MUC8* gene expression via the MEK1/ERK pathway. Furthermore, the activations of RSK1 and CREB are a crucial aspect of the intracellular mechanisms that mediate *MUC8* gene expression in human airway epithelial cells. Molecular cloning of the *MUC8* promoter regulated by various stimuli may yield a deeper insight into ciliated cell differentiation or function.

Acknowledgment—We are deeply grateful to Dr. Jae-Hong Kim for the cDNA construct encoding dominant-negative Raf1 vector.

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Induction of *MUC8* Gene Expression by Interleukin-1β Is Mediated by a Sequential ERK MAPK/RSK1/CREB Cascade Pathway in Human Airway Epithelial Cells

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J. Biol. Chem. 2003, 278:34890-34896.
doi: 10.1074/jbc.M303911200 originally published online July 3, 2003
