Mechanisms of Signal Transduction:

cAMP-responding Element-binding Protein and c-Ets1 Interact in the Regulation of ATP-dependent *MUC5AC* Gene Expression

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Exogenous ATP activates purinoreceptors on the cell surface that regulate diverse cellular functions, including mucous cell secretion in the respiratory epithelium. In this study, ATP increased *MUC5AC* mRNA in primary human nasal epithelial cells and in NCI-H292 pulmonary adenocarcinoma cells *in vitro*. ATP-induced *MUC5AC* mRNA was mediated by phospholipase Cβ3. A dominant-negative mutation in the PDZ binding domain of PLCβ3 inhibited ATP-induced *MUC5AC* gene expression. ATP sequentially activated the phosphorylation of Akt, ERK1/2, p38, RSK1, and cAMP-responding element-binding protein (CREB) in a protein kinase C-independent manner. ATP-induced *MUC5AC* mRNA levels were regulated by CREB via direct interaction with c-Ets1 on the *MUC5AC* gene promoter (located −938 to −930). Effects of CREB and c-Ets1 were additive. Inhibition of either CREB or c-Ets1 inhibited ATP-induced *MUC5AC* gene expression. Stimulation with ATP caused the direct binding of CREB and c-Ets1 to the *MUC5AC* promoter, increasing the phosphorylation of c-Ets1. Chromatin immunoprecipitation assays demonstrated that in the presence of ATP, both c-Ets1 and CREB bound to the *MUC5AC* promoter. The effects of exogenous ATP on *MUC5AC* gene expression are mediated by a complex regulatory cascade controlling interactions between CREB and c-Ets1 that bind to a promoter element in the *MUC5AC* gene enhancing *MUC5AC* gene transcription. ATP-dependent activation of *MUC5AC* gene expression via CREB-c-Ets1 may contribute to mucous cell hypersecretion associated with common respiratory disorders.

Mucus hypersecretion in the airway epithelium is a major characteristic of a number of respiratory diseases, including rhinitis, sinusitis, asthma, chronic obstructive pulmonary disease, and cystic fibrosis (1). Because bacteria, cytokines, and stimulants can interact with host epithelial cells and activate intracellular signaling pathways thereby causing a selective increase in specific *mucin* gene expression and Mucin production, it is important to clarify the molecular mechanism of these interactions. An understanding of the mechanisms that lead to increased mucus secretion in respiratory diseases is critical to improve future therapies. *MUC5AC* is one of the major *mucin* genes in the human respiratory tract, so clarification of the mechanisms regulating *MUC5AC* gene expression is essential for identifying strategies to prevent airway mucus hypersecretion.

Until now, there have been no reports on ATP-induced *MUC5AC* gene expression in the airway, because most of the studies on ATP have taken an electrophysiological approach to determine the role of agonist in inducing Mucin secretion (2, 3). Thus, the mechanism by which ATP can induce *mucin* gene expression in the airway remains unclear. ATP signaling is processed through purinergic receptors. P2Y is a G-protein-coupled receptor, and $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors are highly expressed in NHNE cells (4). P2Y couples to the G-protein subtype, $G\alpha_q$, and the $G\alpha_q$ family activates PLCβ upon stimulation with ligands (5). PLCβ isotypes have a long C-terminal region (~400 residues) that has a relatively low homology among family members (6), and this C-terminal region (residues 903–1142 of PLCβ1) is required for binding and stimulation by $G\alpha_q$ (7). Moreover, PLCβ isotypes are short consensus sequences known as postsynaptic density-95/disc large/ZO-1 (PDZ)-binding motifs. PDZ domains exist in a large number of multifunctional proteins that mediate protein-protein interactions at the postsynaptic density in neurons and junctional complexes in epithelia (8). However, the mechanism of PLCβ3-mediated *MUC5AC* gene expression and the signal molecules involved, especially in the downstream signaling of MAPK,[2] have not yet been demonstrated.

A role for E26 transformation-specific (Ets) *cis*-sequences in the promoter of *MUC5AC* for ATP-induced *MUC5AC* gene expression has not yet been identified. c-Ets1 is a member of a family of transcription factors that play important roles in bio-

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[2] The abbreviations used are: MAPK, mitogen-activated protein kinase; c-Ets1, E26 transformation specific; CREB, cAMP-responding element-binding protein; PLC, phospholipase C; PKC, protein kinase C; RT, reverse transcription; siRNA, small interfering RNA; GST, glutathione *S*-transferase; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; IL, interleukin; CRE, cAMP-response element.

TABLE 1
siRNA sequences for indicated target genes

Gene	siRNA sequence (3′ linker, dTdT)	GenBank™ accession No.
Akt1	GACAACCGCCAUCCAGACU	NM_001014431
ERK1	CAGAUCUUUACAAGCUCUU	NM_002745
p38	CUGUUGACUGGAAGAACAU	NM_001315
RSK1	CUGAUGACACCUUCUACUU	NM_001006665
CREB	UCAAGGAGGCCUUCCUACA	NM_004379
c-Ets1	GCUGACCUCAAUAAGGACA	NM_005238
Control	CCUACGCCACCAAUUUCGU	

logical processes (9, 10). The Ets domain recognizes and binds to a GGAA purine-rich core sequence found in the promoters of specific genes. The interaction between nuclear proteins is essential for transcriptional regulation. Furthermore, protein-protein interactions are involved in every step of cell signaling, including receiving of the signal, selection of the target genes, regulation of DNA binding ability, regulation of transcriptional activity, and turnover of transcription factors (9). We therefore examined the role of c-Ets1 in signal transduction facilitated by interactions with other nuclear transcription factors.

Because increased *MUC5AC* gene expression during inflammation plays an important role in the pathogenesis of airway diseases, we examined whether extracellular ATP up-regulates *MUC5AC* gene expression by activating specific signal transduction pathways. Here, we show that PLCβ3, Akt, MAPK, RSK1, and CREB are sequentially essential for ATP-induced *MUC5AC* gene expression in airway epithelial cells. We also show that CREB mediates the phosphorylation of c-Ets1, which leads to the formation of a protein complex that binds the *MUC5AC* promoter.

EXPERIMENTAL PROCEDURES

Materials—ATP and lipopolysaccharide were purchased from Sigma. All antibodies were purchased from Cell Signaling (Beverly, MA), except for the antibody for c-Ets1, which was from Santa Cruz Biotechnology (Santa Cruz, CA). All of the PLCβ3 isotypes were kindly provided by Dr. Pann-Ghill Suh (Department of Life Science, Pohang University of Science and Technology, Pohang, Korea). PLCβ3 isotypes in pcDNA3.1 were constructed from the cDNA of each of the mutated PLCβ3 isotypes. Each of the individual residues of the PDZ-binding motif (NTQL) of PLCβ3 were mutated to Ala codons (8). The construct encoding c-Ets1 was kindly provided by Dr. Young-Hyuck Im (Biomedical Research Institute, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea). All siRNAs were synthesized by Bioneer (Daejeon, Korea) (Table 1).

Cell Cultures—Passage 2 NHNE cells (2×10^5 cells/well) were seeded in 0.5 ml of culture medium on Transwell clear culture inserts (24.5 mm, 0.45-μm pore size; Costar, Cambridge, MA). Cells were cultured in a 1:1 mixture of bronchial epithelial cell growth medium and Dulbecco's modified Eagle's medium containing all the supplements described previously (12). Cultures were grown while submerged for the first 9 days, during which time the culture medium was changed on day 1 and every other day thereafter. The air-liquid interface was created on day 9 by removing the apical medium and feeding the cultures only from the basal compartment. 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 medium (Invitrogen) supplemented with 10% fetal bovine serum in the presence of penicillin/streptomycin at 37 °C 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 medium with 0.2% fetal bovine serum.

RT-PCR—Real time PCR was performed using an iQ iCycler detection system (Bio-Rad) with iQ SYBR Green Supermix. Reactions were performed in a total volume of 20 μl, which included 10 μl of 2× SYBR Green PCR Master Mix, 300 nM of each primer, and 1 μl of previously reverse-transcribed cDNA template. The following primers were used: *MUC5AC*, forward 5′-CAGCCACGTCCCCTTCAATA-3′ and reverse 5′-ACCG-CATTTGGGCATCC-3′; and β_2-microglobulin, used as a reference for normalization, forward 5′-CGCTCCGTGGCCT-TAGC-3′ and reverse 5′-GAGTACGCTGGATAGCCTCCA-3′. Real time RT-PCR was performed on a MiniOption real time PCR detection system (Bio-Rad). Parameters were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. All reactions were performed in triplicate. The relative quantity of *MUC5AC* mRNA was obtained using the comparative cycle threshold method and was normalized using β_2-microglobulin as an endogenous control.

Western Blot Analysis and Immunoprecipitation—NCI-H292 cells were grown to confluence in 6-well plates. After treatment with ATP, cells were lysed with 2× lysis buffer (250 mM Tris-Cl (pH 6.5), 2% SDS, 4% β-mercaptoethanol, 0.02% bromphenol blue, 10% glycerol). Equal amounts of whole cell lysates were resolved by 10–15% SDS-PAGE and transferred to polyvinylidene difluoride membranes (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. Blots were then incubated overnight with primary antibody in TTBS (0.5% Tween 20 in Tris-buffered saline). After washing with TTBS, blots were further incubated for 45 min at room temperature with anti-rabbit or anti-mouse antibody (Cell Signaling) in TTBS and visualized using the ECL system (GE Healthcare). For immunoprecipitation, cells were washed with ice-cold phosphate-buffered saline, harvested by scraping into lysis buffer (25 mM HEPES, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, protease inhibitor tablet (Complete Mini; Roche Applied Science)), sonicated (four times each for 5 s), and centrifuged at 12,000 × *g* for 15 min. Supernatant lysates (230 μl) were pre-cleared with Gammabind G-Sepharose (GE Healthcare) for 30 min at 4 °C. Following centrifugation, anti-Ets-1 antisera were added to precleared lysates, incubated for 14 h at 4 °C, and microcentrifuged at 4 °C. The pellet was washed three times with lysis buffer.

GST Pulldown Assay—GST fusion proteins were expressed in and purified from bacteria using a glutathione affinity matrix. The GST fusion proteins were eluted from the matrix with glutathione and desalted by centrifugation (Centricon YM-3; Millipore). For interaction assays with cell lysates, the GST fusion protein (250 nM) was incubated with cell lysate (~4 mg of protein/ml) for 1 h at 24 °C in a total volume of 250 μl of buffer (20 mM Tris (pH 7.5), 70 mM NaCl, 1 mM dithiothreitol, 0.6 mM

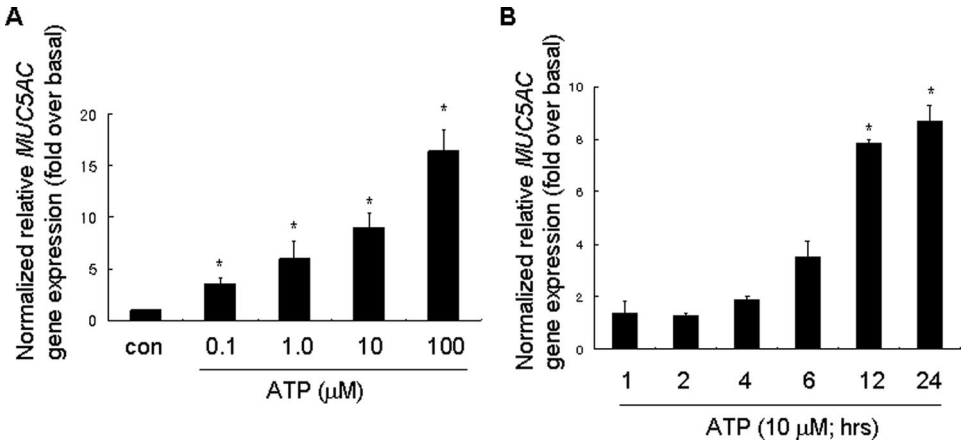


FIGURE 1. **Effect of extracellular ATP on *MUC5AC* gene expression in NHNE cells.** NHNE cells were treated for 24 h with exogenous ATP at the indicated concentrations (*A*) and times (*B*). Cell lysates were harvested for real time quantitative PCR. *, $p < 0.05$ compared with control (vehicle). Data are presented as mean ± S.D. values of three independent experiments. The figures are representative of three independent experiments.

EDTA). A volume of 12.5 μl of packed glutathione-Sepharose slurry was added, and the mixture was rotated at 4 °C for 20 min, after which the affinity matrix was pelleted and washed three times with 500 μl of incubation buffer. Proteins retained on the matrix were solubilized in 2× Laemmli loading buffer and separated by electrophoresis.

Cell Transfection and Luciferase Assays—For luciferase assays, cells were plated in 12-well plates 1 day prior to transfection using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. Approximately 24 h after transfection, cells were treated with ATP for 24 h. For the *MUC5AC* promoter-driven luciferase assay, cells were maintained in serum-free RPMI media for 16–18 h before treatment with ATP. Cells were harvested 24 h after ATP treatment, and the luciferase activity in cell extracts was determined using a luciferase assay system according to the manufacturer's instructions (Promega; Madison, WI). 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 (Upstate). 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) 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 following were the primers used to detect the c-Ets1 element in the *MUC5AC* promoter: forward, GTGTTCATCACACAACAGCACC, and reverse, TCCAGAAATGCTCAGTACCCTCTG. This PCR product did not contain a CRE site. Control primers were for a site ~2,200 bp upstream of c-Ets1 as follows: forward, GCCCCACTGACATAACCACCTGGC, and reverse, GGTCTGACTCGACCAGGTGTAGCC.

Statistical Analysis—The data are presented as the means ± S.D. 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

Extracellular ATP Induces MUC5AC Gene Expression in Normal Human Nasal Epithelial Cells and NCI-H292 Cells—There has been no report on the induction of *MUC5AC* gene expression by extracellular ATP as ATP studies have primarily focused on electrophysiology. We examined whether ATP could induce *mucin* gene expression, because ATP stimulates Mucin secretion, and could participate as an inflammation mediator that can send a signal to nearby cells. First, to examine if ATP increases *MUC5AC* gene expression in normal human nasal epithelial (NHNE) cells, real time PCR analysis was performed. NHNE cells were treated with various doses of ATP for 24 h. Increased *MUC5AC* gene expression occurred in a dose-dependent manner with an EC_{50} value of 9.4 ± 0.15 μM (Fig. 1*A*), and as a result, 10 μM ATP was used for all the subsequent experiments. In addition, *MUC5AC* gene expression increased in a time-dependent manner for up to 12 h after ATP treatment and then reached a plateau (Fig. 1*B*). These results show that extracellular ATP induces *MUC5AC* gene expression in a dose- and time-dependent manner in NHNE cells. When the same experiments (Fig. 1) were performed using NCI-H292 cells, a human lung mucoepidermoid carcinoma cell line, the results were the same (data not shown). Thus, we used NCI-H292 cells for all subsequent molecular experiments.

PLCβ3 and Akt Are Essential for ATP-induced MUC5AC Gene Expression—To determine which molecules are involved in the downstream signaling of $G\alpha_q$ in ATP-induced *MUC5AC* gene expression, we investigated phospholipase C (PLC) β3 (Fig. 2*A*). The phosphorylation of PLCβ3 by ATP reached a maximum at 5 min. Interestingly, there are short consensus sequences known as postsynaptic density-95/discs large/ZO-1 (PDZ)-binding motifs that consist of the amino acids -X(S/T)*X*(V/L)-COOH at the immediate C terminus of the PLCβ3 isotype (13). The PDZ domain of PLCβ3 binds four amino acids of target proteins to the C terminus (8, 14, 15). To identify whether the PDZ domain of the C terminus of PLCβ3 plays an important role in ATP-induced *MUC5AC* gene expression, each of the last four amino acid residues of PLCβ3 (^{1231}NTQL^{1234}-COOH) was mutated to Ala. When treated with ATP, cells transfected with the construct expressing wild-type PLCβ3 showed an increase in *MUC5AC* gene expression, whereas the cells transfected with the construct expressing the dominant-negative mutant PLCβ3 NTQA (L1234A), but not PLCβ3 ATQL (N1231A), NAQL (T1232A), or NTAL (Q1233A), showed a dramatic suppression of *MUC5AC* gene expression (Fig. 2*B*). This result indicates that the Leu residue of the PDZ domain in PLCβ3 is essential for interacting with

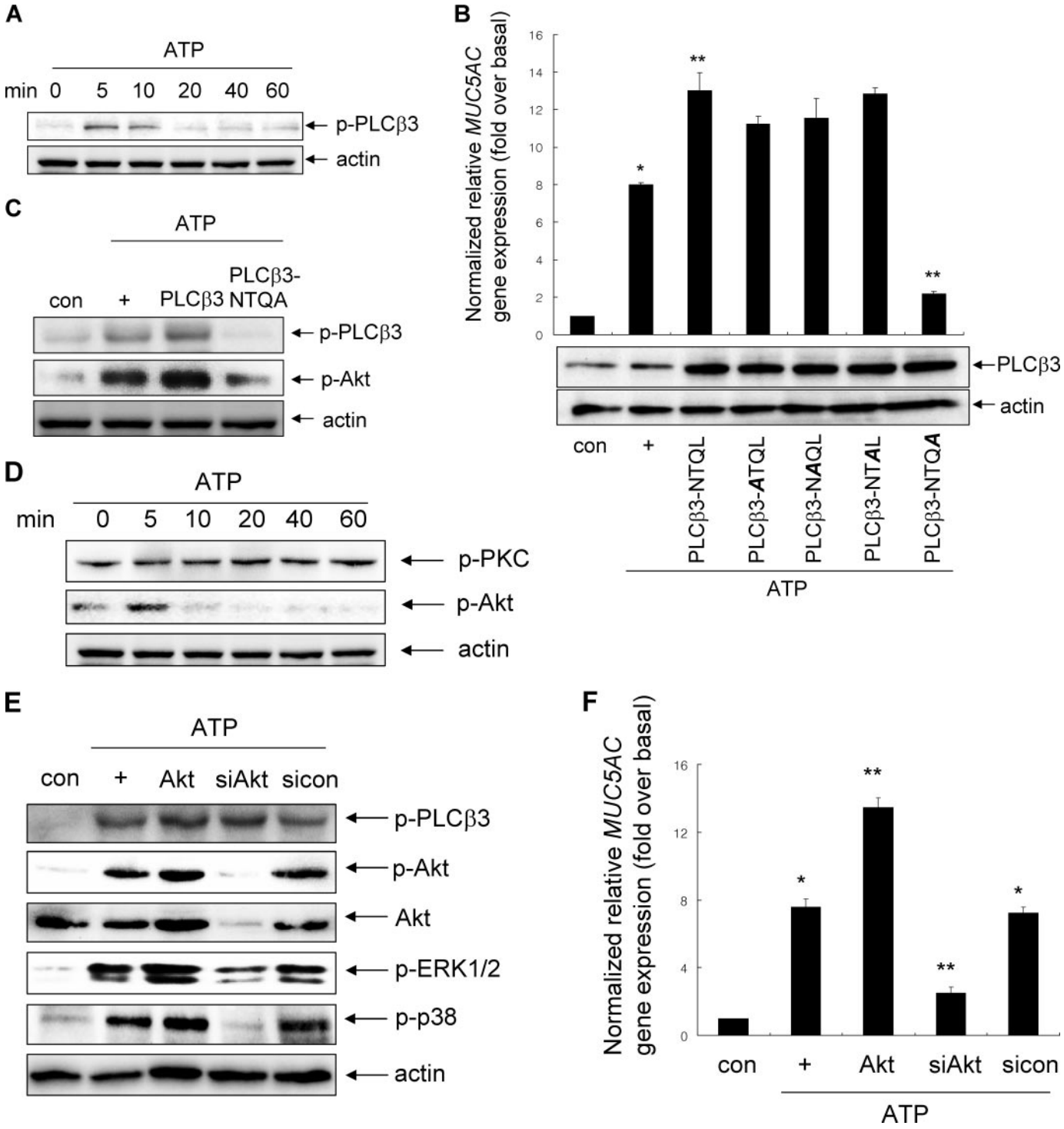


FIGURE 2. **PLCβ3 and Akt, but not PKC, are essential for ATP-mediated *MUC5AC* gene expression.** *A,* confluent and quiescent NCI-H292 cells were treated with ATP (10 μM) for the indicated times, then cell lysates were harvested and analyzed by Western blot using phospho-specific PLCβ3 antibody. Actin was used as a loading control. *B,* cells were transiently transfected with wild-type (^{1231}NTQL^{1234}) or dominant-negative PLCβ3 ATQL (N1231A), NAQL (T1232A), NTAL (Q1233A), or NTQA (L1234A) construct. Each of the individual residues of the PDZ-binding motif (NTQL) of PLCβ3 were mutated to Ala, respectively. Cells were serum-starved overnight and then treated with ATP for 24 h, after which cell lysates were harvested for real time quantitative RT-PCR. *con*, control. *, $p < 0.05$ compared with control (vehicle); **, $p < 0.05$ compared with ATP treatment only. *C,* after cells were transfected with a wild-type PLCβ3 or dominant-negative PLCβ3 NTQA construct, cells were treated with ATP for 5 min prior to the collection of cell lysates for Western blot analysis. Actin was used as a total protein loading control. *D,* quiescent NCI-H292 cells were treated with ATP for the indicated times and then cell lysates were harvested by Western blot using phospho-specific PKC or Akt antibody. Actin was used as a loading control. *E,* cells were transiently transfected with a wild-type or siRNA construct of Akt. Cells were treated with ATP for 5 min, after which cell lysates were harvested for Western blot analysis using phospho-specific antibodies. *F,* after cells were transfected with a wild-type or siRNA construct of Akt, cells were treated with ATP for 24 h prior to the collection of total RNA for real time quantitative RT-PCR. *, $p < 0.05$ compared with control (vehicle); **, $p < 0.05$ compared with ATP treatment only. The figures are representative of three independent experiments.

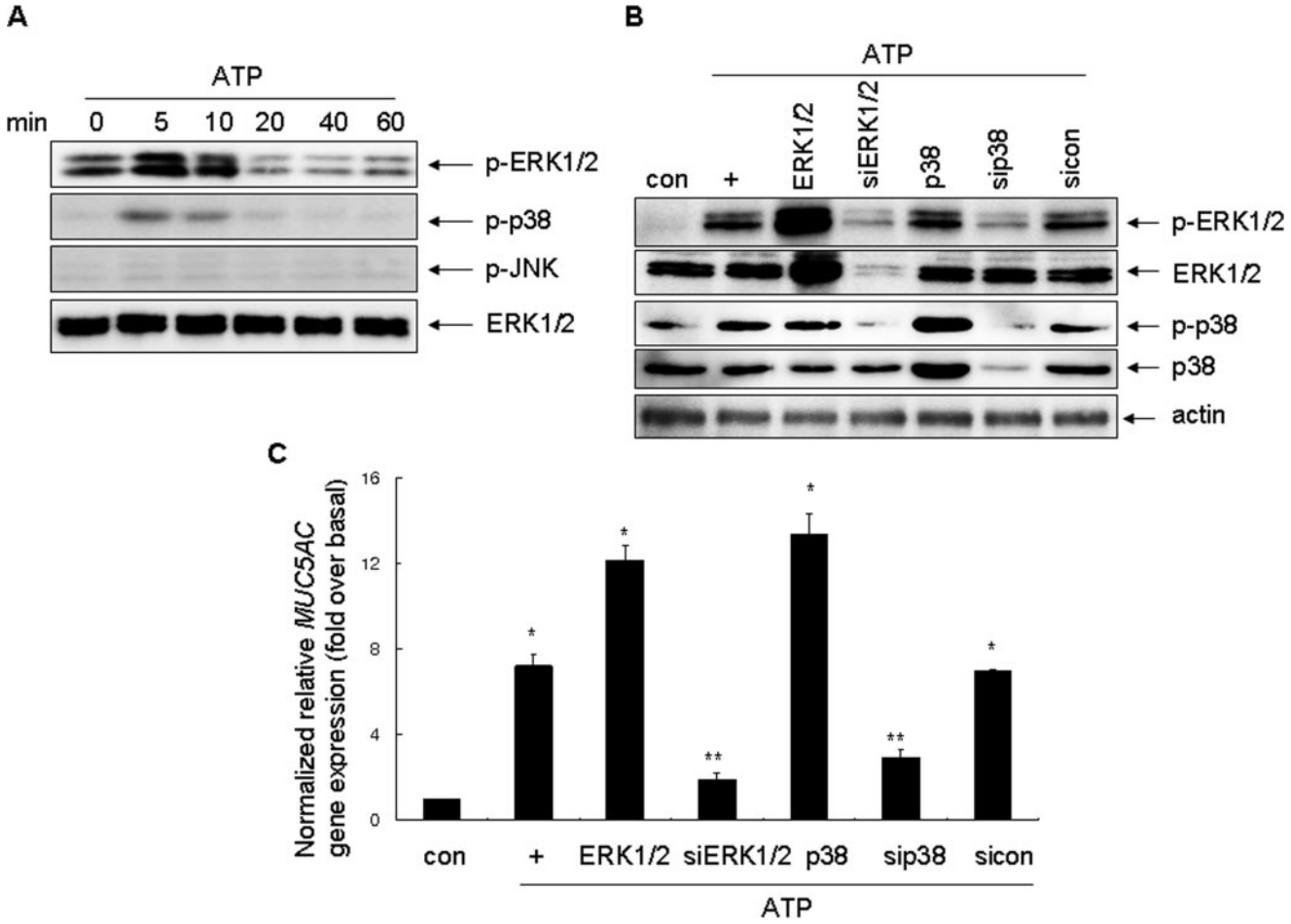


FIGURE 3. **ERK1/2 and p38 MAPKs are essential for ATP-mediated *MUC5AC* gene expression.** *A,* confluent and quiescent NCI-H292 cells were treated with ATP for the indicated times, and then cell lysates were harvested and analyzed by Western blot using phospho-specific antibodies. *B,* cells were transiently transfected with wild-type or siRNA construct of ERK1/2 and p38 MAPKs, respectively. Cells were serum-starved overnight and then treated with ATP (10 μM) for 5 min or 24 h, after which cell lysates were harvested for Western blot analysis or real time quantitative RT-PCR (*C*). Actin was used as a total protein loading control. *, $p < 0.05$ compared with control (vehicle); **, $p < 0.05$ compared with wild-type construct treated by ATP. *con,* control. The figures are representative of three independent experiments.

some target signaling molecule(s) to induce *MUC5AC* gene expression after ATP treatment. In addition, phosphorylation of PLCβ3 and Akt were suppressed when cells were transfected with the PLCβ3 NTQA dominant-negative construct (Fig. 2*C*). Even though many reports have shown that PLCβ3 regulates PKC activation, it appears that PKC is not involved in ATP-induced signaling (Fig. 2*D*). Interestingly, whereas PKC phosphorylation was not altered by ATP, Akt phosphorylation reached a maximum at 5 min. This result may indicate that ATP signaling is mediated through PLCβ3 and Akt. To examine the role of Akt in ATP-induced *MUC5AC* gene expression in our system, we used both wild-type Akt and siRNA-Akt constructs. Wild-type Akt activated the phosphorylation of Akt, ERK1/2, and p38 MAPK, whereas an siRNA-Akt construct inhibited the phosphorylation of Akt, ERK1/2, and p38 MAPK but not PLCβ3. In addition, Akt levels were increased in cells transfected with construct expressing wild-type Akt and decreased in cells transfected with siRNA-Akt. However, *MUC5AC* gene expression increased when cells were transfected with wild-type Akt compared with treatment with ATP alone (Fig. 2*F*), and *MUC5AC* gene expression was significantly suppressed by siRNA-Akt. These results show that PLCβ3 and Akt appear to function together in a pathway mediating ATP-induced *MUC5AC* gene expression and Akt functions downstream of PLCβ3 in NCI-H292 cells.

Both ERK1/2 and p38 MAPKs Are Essential for ATP-induced MUC5AC Gene Expression—We also investigated whether the ERK1/2 or p38 pathways are involved in ATP-induced *MUC5AC* gene expression. The phosphorylation of ERK1/2 and p38 peaked at 5 min after ATP treatment (Fig. 3*A*), and no change in phosphorylation of JNK was observed. To investigate the possible involvement of ERK1/2 and p38 MAPKs in ATP-induced *MUC5AC* gene expression, either the siRNA-ERK1/2 or siRNA-p38 construct was applied before treatment with ATP. Western blot analysis showed that the siRNA-ERK1/2 and siRNA-p38 constructs clearly inhibit ERK1/2 and p38 MAPK, respectively (Fig. 3*B*). We also examined the transcript level of *MUC5AC* by performing real time PCR analysis (Fig. 3*C*). Whereas ATP-induced *MUC5AC* gene expression was higher in cells transfected with wild-type constructs compared with cells treated with ATP alone, *MUC5AC* gene expression was significantly suppressed by both siRNA constructs. These results suggest that both ERK1/2 and p38 MAPKs may be downstream signaling proteins of Akt and may be essential for ATP-induced *MUC5AC* gene expression.

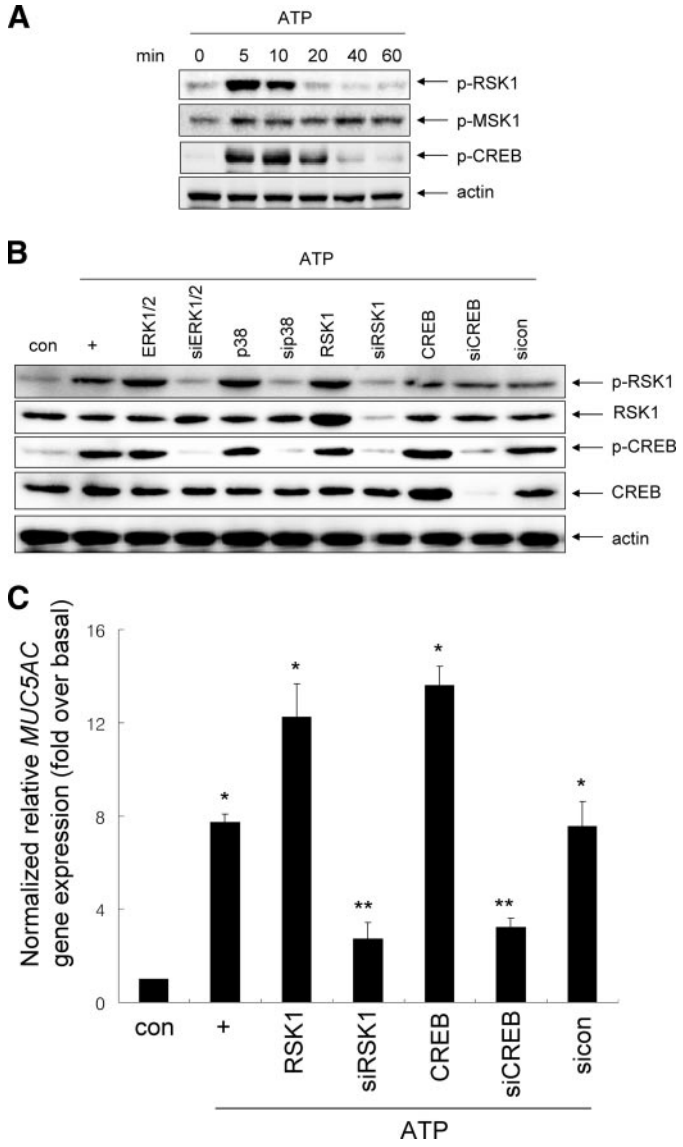


FIGURE 4. **RSK1 and CREB, but not MSK1, act as downstream signaling mediators of ERK1/2 and p38 MAPKs to induce ATP-mediated *MUC5AC* gene expression.** *A,* confluent and quiescent NCI-H292 cells were treated with ATP for the indicated times, and then cell lysates were analyzed by Western blot using phospho-specific antibodies. *B,* cells were transiently transfected with wild-type or siRNA construct of ERK1/2, p38, RSK1, and CREB, respectively. Cells were serum-starved overnight and then treated with ATP for 5 min or 24 h, after which cell lysates were harvested for Western blot analysis or real time quantitative RT-PCR (*C*). Actin was used as a total protein loading control. *, $p < 0.05$ compared with control (vehicle); **, $p < 0.05$ compared with wild-type construct treated by ATP. *con,* control. These figures are representative of three independent experiments.

ATP-induced MUC5AC Gene Expression Is Mediated by RSK1 and CREB Sequentially but Not by MSK1—To determine which molecules are involved in the nuclear signaling of ATP-induced *MUC5AC* gene expression, we examined mitogen- and stress-activated protein kinase (MSK) 1, p90 ribosomal S6 protein kinase (RSK) 1, and cAMP-response element-binding protein (CREB), which are all associated with calcium activation. As seen in Fig. 4*A*, alterations were seen in the phosphorylation status of RSK1 and CREB after ATP treatment, but no changes were observed for MSK1. RSK1 phosphorylation peaked at 5 min and then decreased back to base line 20 min after ATP treatment. This is in contrast to findings for other proinflammatory mediators like IL-1β or tumor necrosis factor-α, where RSK1 peaked 30 min after treatment (16), demonstrating that phosphorylation of RSK1 occurs more rapidly when stimulated by ATP.

To examine the signaling molecules downstream of RSK1, we investigated CREB, a well known downstream molecule of RSK1. Transient phosphorylation of CREB was observed upon stimulation with ATP, reaching a maximum peak at 5–10 min. These results show the possible involvement of RSK1 and CREB in ATP-induced signaling during inflammation. To confirm the role of RSK1 and CREB at the cellular level, we used constructs expressing wild-type ERK1/2, p38, RSK1, and CREB, and we also used specific siRNAs for these proteins. Interestingly, inhibition of either ERK1/2 or p38 MAPK decreased RSK1 phosphorylation (Fig. 4*B*), suggesting that RSK1 activity is simultaneously controlled by both ERK1/2 and p38 MAPKs and that RSK1 may act as a downstream signaling molecule of these MAPKs. In addition, CREB phosphorylation was decreased by siRNA-RSK1. These results suggest that RSK1 acts upstream of CREB. Furthermore, siRNA-RSK1 and siRNA-CREB constructs also decreased the steady-state level of *MUC5AC* mRNA expression in NCI-H292 cells (Fig. 4*C*). These results indicate that RSK1 and CREB are controlled by ERK1/2 and p38 MAPKs, sequentially, and may be involved in ATP-induced *MUC5AC* gene expression in the nucleus.

cis-Acting Regulatory Element, c-Ets1, Mediates ATP-induced MUC5AC Transcriptional Activity—To identify the ATP-responsive region within the *MUC5AC* promoter, cells were transiently transfected with various deletion mutants and treated with ATP for 24 h. ATP selectively increased the luciferase activity of the −950/−1 region of the *MUC5AC* promoter. ATP-induced luciferase activity of the −929/−1 region of the *MUC5AC* promoter was lower than that of the −950/−1 regions, indicating that the −950/−929 region of the *MUC5AC* promoter may be necessary for its response to ATP (Fig. 5*A*). Interestingly, there is no CRE site in the −950/−929 region. This finding suggests that a transcription factor other than CREB may have a crucial role. To identify the ATP-responsive *cis*-element in the *MUC5AC* promoter, we used the TFSEARCH version 1.3 data base. This search strategy identified a candidate, c-Ets1 (score = 91.2; threshold, 90.0). To date, more than 25 mammalian Ets family members have been characterized and are known to control important biological processes, including cellular proliferation, differentiation, lymphocyte development and activation, and transformation by recognizing a GCA core motif in the promoter or enhancer of their target genes (9). To further investigate whether c-Ets1, which was found within the −938/−930 region of the *MUC5AC* promoter, might act as a *cis*-element, three constructs for selective mutagenesis of the c-Ets1-binding site were generated. Only the −932 mutant construct decreased the responsiveness of the wild-type *MUC5AC* promoter construct (Fig. 5*B*). However, neither the −935 nor −938 mutant constructs were able to suppress luciferase activity. These results suggest that c-Ets1 (−938/−930 region) in the regulatory region of the *MUC5AC* promoter may be critical for ATP-induced up-regulation of *MUC5AC* transcriptional activity. To

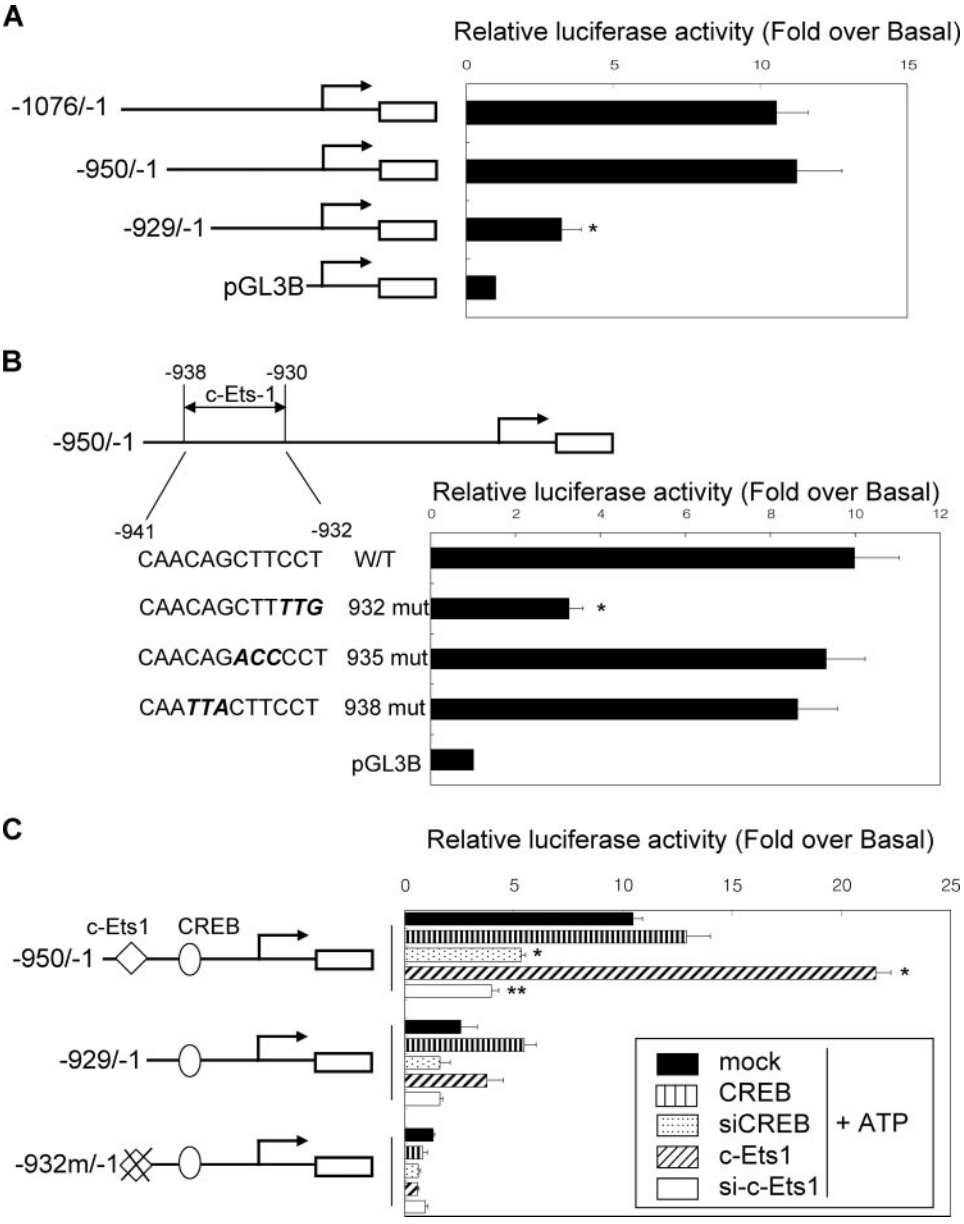
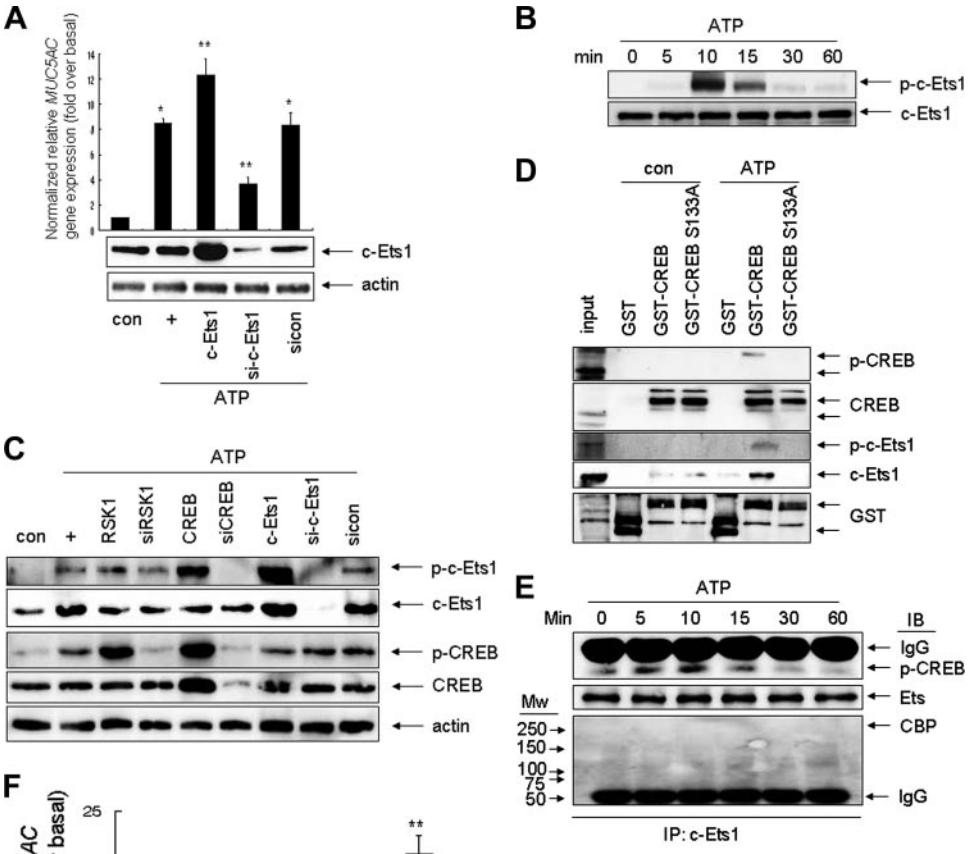


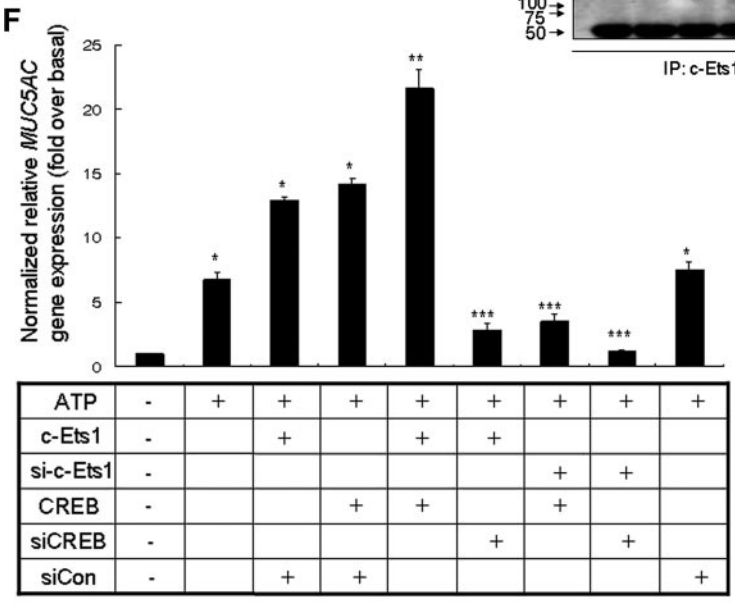
FIGURE 5. **ATP-induced c-Ets1 activation mediates *MUC5AC* transcription via the *cis*-acting c-Ets1 regulatory motif.** *A,* cells were transiently transfected with several *MUC5AC* promoter luciferase reporter constructs and stimulated with ATP (10 μM) for 24 h. Cell lysates were harvested and reporter assays were performed according to the manufacturer's instructions (see "Experimental Procedures"). *, $p < 0.05$ compared with −1076/−1 reporter construct. *B,* site-directed selective mutagenesis was performed to construct c-Ets1-binding site mutants as indicated. Luciferase activities are shown after correction for transfection efficiency using the β-galactosidase activity of the cell lysates. Values shown are means ± S.D. of experiments performed three or more times. *, $p < 0.05$ compared with wild-type reporter construct. *C,* cells were transiently transfected with a −950/−1, −929/−1, or −932m/−1 reporter constructs in combination with either wild-type or siRNA constructs for CREB or c-Ets1. Cells were stimulated with ATP (10 μM) for 24 h. Cell lysates were harvested and reporter assays performed according to the manufacturer's instructions. *, $p < 0.05$ compared with ATP treatment; **, $p < 0.05$ compared with wild-type c-Ets1 treated by ATP. These figures are representative of three independent experiments.

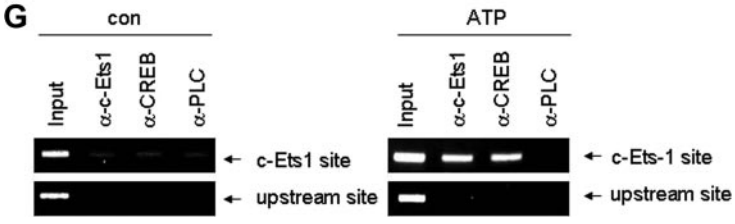
investigate whether c-Ets-mediated activity was affected by CREB, we co-transfected the −950/−1 construct as a wild type, a −929/−1 construct that did not contain a c-Ets1 site, or a −932m construct as a site-directed mutation of c-Ets1 site with either wild-type CREB, siRNA-CREB, wild-type c-Ets1, or siRNA-c-Ets1, respectively. The activity of the −950/−1 construct was not affected by wild-type CREB but was decreased in the presence of siRNA-CREB. In addition, the activity of the −950/−1 construct increased with the wild-type c-Ets1 but decreased with the siRNA-c-Ets1. However, the activity of the −929/−1 construct only slightly increased with wild-type CREB and decreased with the siRNA-CREB. The −932m construct activity was not affected by these genes. These results suggest that the activity of the −950/−1 construct is mainly regulated by c-Ets and is partially affected by CREB.

c-Ets1 Up-regulates ATP-induced MUC5AC Gene Expression through Interaction with CREB—To examine whether c-Ets1 affects ATP-induced *MUC5AC* gene expression, transient transfection with a plasmid construct encoding either wild-type c-Ets1 or siRNA-c-Ets1 was performed (Fig. 6*A*). Whereas wild-type c-Ets1 up-regulated ATP-induced *MUC5AC* gene expression, siRNA-c-Ets1 attenuated *MUC5AC* gene expression, demonstrating that c-Ets1 may play an important role in ATP-induced *MUC5AC* gene expression. Next, to further investigate the effect of ATP on c-Ets1 activity, cells were treated with ATP in a time-dependent manner, and c-Ets1 phosphorylation was analyzed by Western blot analysis using a specific antibody against phosphorylated c-Ets1 (Ser(P)-282). c-Ets1 was rapidly phosphorylated 10 min after ATP treatment, and this persisted for up to 15 min (Fig. 6*B*). In addition, ATP could induce CREB and c-Ets1 activation in NHNE cells and *in vivo* (data not shown). Furthermore, to determine the effect of RSK1 or CREB on the phosphorylation of c-Ets1, we used both wild-type and siRNA constructs of RSK1, CREB, and c-Ets1 (Fig. 6*C*). Interestingly, siRNA-CREB suppressed the phosphorylation of c-Ets1, whereas siRNA of c-Ets1 had no effect the phosphorylation of CREB. In addition, RSK1 did not affect the phosphorylation of c-Ets1. These results suggest that CREB may activate c-Ets1 in an RSK1-independent manner. Furthermore, we investigated the relationship between c-Ets1 and CREB in ATP-induced *MUC5AC* gene expression because both c-Ets1 and CREB are transcription factors, and c-Ets1 activity is selectively regulated by interactions with different transcription factors such as AP-1, NF-κB, and

Pax family members (9). To explore the interaction between c-Ets1 and CREB using *in vitro* binding assays, we generated wild-type CREB and mutant CREB (S133A) as GST fusion proteins. These GST fusion proteins were incubated with NCI-H292 cell lysates obtained after stimulation with ATP for 10 min. As shown in Fig. 6*D*, CREB interacted with c-Ets1 and activated the phosphorylation of c-Ets1, whereas CREB S133A did not interact with c-Ets1. Furthermore, cell extracts obtained after stimulation with ATP were immunoprecipitated with anti-c-Ets1 and then blotted with the indicated antibodies. Complex formation between c-Ets1 and CREB, but not c-Ets1 and CBP, was observed 5–10 min after exposure to ATP (Fig. 6*E*). These results are noteworthy because several reports have shown that c-Ets1 can bind to CBP/p300 but not to CREB (10, 17, 18). Thus, our results suggest that CREB may play a role in regulating the basal transcriptional activation activity of c-Ets1. Moreover, both c-Ets1 and CREB have an additive effect on the induction of *MUC5AC* gene expression, and inhibition of either CREB or c-Ets1 suppressed ATP-induced *MUC5AC* gene expression (Fig. 6*F*), indicating that both CREB and c-Ets1 are necessary for ATP-induced *MUC5AC* transcription. To further confirm whether c-Ets1 and CREB interact with the c-Ets1 site in the *MUC5AC* promoter, we performed chromatin immunoprecipitation experiments. Chromatin was prepared from ATP-treated cells and then immunoprecipitated with an anti-c-Ets1, CREB, or PLC antibody as a nonrelevant antibody. PCR was performed on immunoprecipitated DNA after reversal of cross-linking using primers specific for the c-Ets1 recognition site. The PCR product did not contain a CRE site (−851 to −844). The control primers were for a site ~2,200 bp upstream of c-Ets1. As shown in Fig. 6*G*, the c-Ets1 site was specifically immunoprecipitated with the anti-c-Ets1 and CREB antibodies. This suggests that c-Ets1 interacts selectively with CREB at the c-Ets1 site of the *MUC5AC* promoter. Taken together, these findings suggest that ATP leads to enhanced recruitment of c-Ets1 by CREB to augment *MUC5AC* transcriptional activation.







DISCUSSION

ATP is tightly regulated and its extracellular concentration is kept low by ecto-ATP/ADPases (CD39) (19). Intracellular ATP concentra-

tions are in the range of 5–10 mM, and cellular responsiveness to a pathogen increases the amount of ATP in the pericellular space by exocytosis (20, 21). Thus, the concentration of ATP affects physiological cellular homeostasis. Because most of the literature on the role of ATP in inflammation is focused on the secretion of inflammatory mediators such as eosinophil cationic protein, radical oxygen intermediates, and IL-8 (22, 23), the effect of extracellular ATP on *mucin* gene expression during inflammation remains a matter of speculation. In particular, there have been no reports on ATP-induced *MUC5AC* gene expression in the airway until now, although UTP was found to induce gene expression of *MUC5B*, but not *MUC5AC,* in human tracheobronchial epithelial cells (24). Accordingly, we hypothesized that extracellular ATP responses may provide additional insight into Mucin production during inflammation. First, we investigated the effect of ATP on *MUC5AC* gene expression in normal human nasal epithelial cells. As shown in Fig. 1, ATP increased *MUC5AC* gene expression in NHNE and NCI-H292 cells in a dose- and time-dependent manner.

Understanding the biochemical characteristics of the PDZ domain in PLCβ will provide additional insight into the molecular signaling mechanism that leads to protein complex formation. Although previous studies have reported that key amino acids of the PDZ domain, namely PLCβ3, Thr-1232, and Leu-1234, are involved in the binding of Na^+/H^+ exchanger regulatory factor 2 in COS7 cells, and the binding of Shank2 during mGluR-mediated Ca^{2+} signaling in HN33 hippocampal cells (8, 15), our results show that the binding activity of the PDZ domain, and consequently the function of PLCβ3, is determined by Leu-1234 in airway epithelial cells. Although most studies report that $G\alpha_q$- protein activates PLCβ isoforms, which in turn activate PKC, our study shows that, at least in the airway epithelium, Akt, rather than PKC, is activated during ATP signaling. This result is consistent with the report that there is direct interaction between PLC-dependent signaling and the activation of Akt in astrocytoma cells (25). They demonstrated that G-protein-coupled receptor-mediated activation of Akt was inhibited by wortmannin, suggesting that phosphatidylinositol 3-kinase appears to function in this pathway but not PKC. They also demonstrated that Akt has been activated by direct activation of the G-protein $\beta\gamma$ subunit-sensitive phosphatidylinositol 3-kinase γ and p110β isoforms (25). These observations indicate that PKC may not be involved in the ATP-stimulated signaling pathway in the airway epithelial cells. Furthermore, we also examined how extracellular ATP induces *MUC5AC* gene expression in the nucleus. In our previous studies, MSK1 and RSK1 were identified as intranuclear molecules involved in cytokine-induced *mucin* gene expression (16, 26). Because these proteins are tightly regulated by MAPK in NHNE cells, their effects on ATP-mediated proinflammatory signaling also need to be elucidated. As shown in Fig. 4, RSK1 is involved in the ATP-mediated signaling pathway, but MSK1 is not, suggesting that the signaling pathways leading to *MUC5AC* gene expression are distinct and depend on the type of stimuli and cell lines used. The question of what molecule could mediate this signaling event in the nucleus then arises. We examined CREB because it is known to be regulated by RSK1 (16). We demonstrated that ATP-activated RSK1 is required for activation of CREB (Fig. 4*B*). Interestingly, even though CREB is involved in the ATP signaling pathway, the −950/−929 region of the *MUC5AC* promoter does not have a CREB-binding (CRE) site. In our previous article (26), CRE-mediated luciferase activity, induced by either IL-1β or tumor necrosis factor-α (−929/−1 construct), was increased by about 3-fold compared with basal activity. In the same study, the activity of the −1376/−1 construct decreased luciferase activity to basal levels, which suggested the presence of another transcription factor in the −1376/−929 area acting as a suppressor. In this experiment, although the CRE site (−851 to −844) did not play an important role in ATP-induced *MUC5AC* transcription, it may have an additive or synergic effect as a result of interaction with another transcription factor to induce complete expression of *MUC5AC* (Fig. 5, *A* and *C*). To search for such factor(s), we used a 22-bp sequence (−950 to −929) with a high degree of stringency and identified c-Ets1.

In our previous study (26), we demonstrated that CREB is essential for proinflammatory cytokines-induced *MUC5AC* gene expression. However, it seemed that CREB was not sufficient for fully cytokine-induced *MUC5AC* gene expression. A transcription factor(s) other than CREB is required for maximal ATP–induced expression of the *MUC5AC* gene in airway epithelium. As seen in Fig. 5, after analyzing the −950/−929 region of the *MUC5AC* promoter, it appears that c-Ets1 may play a role in ATP-induced *MUC5AC* transcription. The consensus sequence for c-Ets1 is PuC(C/A)GGA(A/T)GCPy (GGA(A/T), core sequence) (27, 28). In this consensus sequence, the GG of the core sequence in the c-Ets1-binding site may be critical for inducing *MUC5AC* gene expression after

FIGURE 6. **CREB can bind and regulate the phosphorylation of c-Ets1 in ATP-induced *MUC5AC* gene expression.** *A,* cells were transiently transfected with wild-type or siRNA constructs of c-Ets1. Cells were serum-starved and treated with ATP (10 μM) for 24 h, after which cell lysates were harvested for Western blot analysis and real time PCR. *, $p < 0.05$ compared with control; **, $p < 0.05$ compared with ATP treatment. *B,* confluent and quiescent cells were treated with ATP (10 μM) for the indicated times, and then cell lysates were harvested and analyzed by Western blot using phospho-specific c-Ets1 antibody (Ser-282). Total c-Ets1 was used as a loading control (*con*). *C,* cells were transiently transfected with a wild-type or siRNA construct for RSK1, CREB, or c-Ets1. The cells were then serum-starved overnight and treated with ATP for 10 min, after which cell lysates were harvested for Western blot analysis. *D,* an *in vitro* GST pulldown assay was performed using bacterially expressed GST-wild-type CREB and CREB S133A proteins as exogenous binding partners. Lysates were prepared from NCI-H292 cells treated by ATP for 10 min, and 1 mg of lysate was incubated with 250 nM GST fusion proteins, and the membrane transfers of bound proteins were probed with the indicated antibodies. *E,* confluent and quiescent cells were treated for the indicated times (min) with ATP. Total cell lysates were then immunoprecipitated with anti-c-Ets1 antibody and blotted with several antibodies. *IP,* immunoprecipitation; *IB,* immunoblotting. *F,* cells were transiently transfected with a wild-type or siRNA constructs of c-Ets1 or CREB, and cells were then serum-starved and treated with ATP (10 μM) for 24 h, after which cell lysates were harvested for real time PCR. *, $p < 0.05$ compared with control; **, $p < 0.05$ compared with ATP treatment; ***, $p < 0.05$ compared with wild-type c-Ets1 and wild-type CREB transfectants. *G,* chromatin prepared from cells with/without ATP treatment was immunoprecipitated using several antisera, and PCR was performed on DNA purified from input chromatin (*Input*) or immunoprecipitated chromatin, using primer pairs surrounding the c-Ets1 site (did not contain CRE site) or an upstream 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. Distilled water was used a solvent of ATP as a vehicle control. These figures are representative of three independent experiments.

exposure of ATP (Fig. 5*B*). The c-Ets1 recognition sequence, which exists at the −938/−930 position in the *MUC5AC* promoter, is recognized by the Ets family of transcription factors that share a conserved 85-amino acid DNA binding domain known as the Ets domain (9). Recently, there has been controversy with the role(s) of the Ets factor, because Ets-1 has dual functions to regulate cellular phenomena as follows: Ca^{2+}-dependent phosphorylation of Ets1 results in the loss of DNA binding activity in murine T cell hybridoma Hd11.2 cells (29), whereas Ets factor PEA3 can transactivate the proximal region of the *MUC4* promoter and increase *MUC4* mRNA levels in pancreatic cancer cell lines (CAPAN-1 and -2) (30). This discrepancy may due to differences in the type of stimuli or cell lines used. In this study, both c-Ets1 and CREB had an additive effect on the induction of *MUC5AC* gene expression (Fig. 6*F*), showing that both CREB and c-Ets1 play an important role in ATP-induced *MUC5AC* transcription. These results suggest three possibilities as follows: 1) c-Ets1 may be a key factor and may form a ternary complex for *MUC5AC* gene expression; 2) c-Ets1 may be a cofactor recruited by a major transcription factor such as CREB; or 3) c-Ets1 may activate its own pathway. Li *et al.* (9) reported that there is a stable interaction between c-Ets1 and other transcription factors, such as AP-1, NF-κB, and Pax family members in response to diverse signals including cytokines, growth factor, and cellular stresses. In Fig. 6*C*, c-Ets1 did not affect ATP-induced phosphorylation of CREB, and RSK1 did not affect ATP-induced phosphorylation of c-Ets1. Only CREB enhanced the phosphorylation of c-Ets1, indicating that CREB can regulate c-Ets1 phosphorylation, but in an RSK1-independent manner.

More interestingly, many reports have shown that c-Ets1 can bind to CBP/p300, but not CREB, and these authors have suggested that CBP/p300 might mediate interactions between c-Ets1 and other transcription factors (19–21). Even though c-Ets1 and CREB are critical transcription factors for erythroid differentiation (31) and human Cdc212 transcription in human melanoma cell line A375 cells (11), there was no evidence whether c-Ets1 may bind to CREB directly. In our system, GST pulldown, immunoprecipitation, and ChIP assays revealed that CREB could induce the phosphorylation of c-Ets1, and the interaction between CREB and c-Ets1 had a synergistic effect on ATP-induced *MUC5AC* gene expression by docking CREB at the c-Ets1 site of the *MUC5AC* promoter (Fig. 6). Taken together, these findings demonstrate that CREB may interact directly with the c-Ets1 transcription factor, which is thought to function as a bridging protein between DNA-binding transcription factors and basal transcription factors, thereby integrating diverse signaling pathways involved in regulating *MUC5AC* gene expression.

REFERENCES

1. Rose, M. C., and Voynow, J. A. (2006) *Physiol. Rev.* **86,** 245–278
2. Kreda, S. M., Okada, S. F., van Heusden, C. A., O'Neal, W., Gabriel, S., Abdullah, L., Davis, C. W., Boucher, R. C., and Lazarowski, E. R. (2007) *J. Physiol.* (*Lond.*) **584,** 245–259
3. Zhu, Y., Ehre, C., Abdullah, L. H., Sheehan, J. K., Roy, M., Evans, C. M., Dickey, B. F., and Davis, C. W. (2007) *J. Physiol.* (*Lond.*) **586,** 1977–1992
4. Kim, C. H., Kim, S. S., Choi, J. Y., Shin, J. H., Kim, J. Y., Namkung, W., Lee, J. G., Lee, M. G., and Yoon, J. H. (2004) *Am. J. Physiol.* **287,** L835–L842
5. Berstein, G., Blank, J. L., Smrcka, A. V., Higashijima, T., Sternweis, P. C., Exton, J. H., and Ross, E. M. (1992) *J. Biol. Chem.* **267,** 8081–8088
6. Jhon, D. Y., Lee, H. H., Park, D., Lee, C. W., Lee, K. H., Yoo, O. J., and Rhee, S. G. (1993) *J. Biol. Chem.* **268,** 6654–6661
7. Kim, C. G., Park, D., and Rhee, S. G. (1996) *J. Biol. Chem.* **271,** 21187–21192
8. Hwang, J. I., Heo, K., Shin, K. J., Kim, E., Yun, C., Ryu, S. H., Shin, H. S., and Suh, P. G. (2000) *J. Biol. Chem.* **275,** 16632–16637
9. Li, R., Pei, H., and Watson, D. K. (2000) *Oncogene* **19,** 6514–6523
10. Jayaraman, G., Srinivas, R., Duggan, C., Ferreira, E., Swaminathan, S., Somasundaram, K., Williams, J., Hauser, C., Kurkinen, M., Dhar, R., Weitzman, S., Buttice, G., and Thimmapaya, B. (1999) *J. Biol. Chem.* **274,** 17342–17352
11. Feng, Y., Goulet, A. C., and Nelson, M. A. (2004) *Gene* (*Amst.*) **330,** 75–84
12. Yoon, J. H., Kim, K. S., Kim, S. S., Lee, G. J., and Park, I. Y. (2000) *Ann. Otol. Rhinol. Laryngol.* **109,** 594–601
13. Kornau, H. C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995) *Science* **269,** 1737–1740
14. Sheng, M., and Sala, C. (2001) *Annu. Rev. Neurosci.* **24,** 1–29
15. Hwang, J. I., Kim, H. S., Lee, J. R., Kim, E., Ryu, S. H., and Suh, P. G. (2005) *J. Biol. Chem.* **280,** 12467–12473
16. Song, K. S., Seong, J. K., Chung, K. C., Lee, W. J., Kim, C. H., Cho, K. N., Kang, C. D., Koo, J. S., and Yoon, J. H. (2003) *J. Biol. Chem.* **278,** 34890–34896
17. Yang, C., Shapiro, L. H., Rivera, M., Kumar, A., and Brindle, P. K. (1998) *Mol. Cell. Biol.* **18,** 218–2229
18. Foulds, C. E., Nelson, M. L., Blaszczak, A. G., and Graves, B. J. (2004) *Mol. Cell. Biol.* **24,** 10954–10964
19. Idzko, M., Hammad, H., van Nimwegen, M., Kool, M., Willart, M. A., Muskens, F., Hoogsteden, H. C., Luttmann, W., Ferrari, D., Di Virgilio, F., Virchow, J. C., Jr., and Lambrecht, B. N. (2007) *Nat. Med.* **13,** 913–919
20. Coade, S. B., and Pearson, J. D. (1989) *Circ. Res.* **65,** 531–537
21. Di Virgilio, F., Chiozzi, P., Ferrari, D., Falzoni, S., Sanz, J. M., Morelli, A., Torboli, M., Bolognesi, G., and Baricordi, O. R. (2001) *Blood* **97,** 66–71
22. Idzko, M., Panther, E., Bremer, H. C., Sorichter, S., Luttmann, W., Virchow, C. J., Jr., Di Virgilio, F., Herouy, Y., Norgauer, J., and Ferrari, D. (2003) *Br. J. Pharmacol.* **138,** 1244–1250
23. Ferrari, D., la Sala, A., Panther, E., Norgauer, J., Di Virgilio, F., and Idzko, M. (2006) *J. Leukocyte Biol.* **79,** 7–15
24. Chen, Y., Zhao, Y. H., and Wu, R. (2001) *Am. J. Respir. Cell Mol. Biol.* **25,** 409–417
25. Tang, X., Batty, I. H., and Downes, C. P. (2002) *J. Biol. Chem.* **277,** 338–344
26. Song, K. S., Lee, W. J., Chung, K. C., Koo, J. S., Yang, E. J., Choi, J. Y., and Yoon, J. H. (2003) *J. Biol. Chem.* **278,** 23243–23250
27. Nye, J. A., Petersen, J. M., Gunther, C. V., Jonsen, M. D., and Graves, B. J. (1992) *Genes Dev.* **6,** 975–990
28. Dittmer, J. (2003) *Mol. Cancer* **2,** 29
29. Cowley, D. O., and Graves, B. J. (2000) *Genes Dev.* **14,** 366–376
30. Fauquette, V., Perrais, M., Cerulis, S., Jonckheere, N., Ducourouble, M. P., Aubert, J. P., Pigny, P., and Van Seuningen, I. (2005) *Biochem. J.* **386,** 35–45
31. Marziali, G., Perrotti, E., Ilari, R., Lulli, V., Coccia, E. M., Moret, R., Kühn, L. C., Testa, U., and Battistini, A. (2002) *Oncogene* **21,** 7933–7944