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

Received for publication, April 1, 2008, and in revised form, July 22, 2008 Published, JBC Papers in Press, August 1, 2008, DOI 10.1074/jbc.M802507200

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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 CB2. A dominant-negative mutation in the PDZ binding domain of PLCβ3 inhibited ATP-induced MUC5AC gene expression. ATP sequentally 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.

Mucous 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 stimuliants 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 mechanisms of these interactions. An understanding of the mechanisms that lead to increased mucous 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 P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 receptors are highly expressed in NHNE cells (4). P2Y couples to the G-protein subtype, Goq, and the Goq 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 Goq (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, 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-

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.
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 isoforms were kindly provided by Dr. Pann-Ghill Suh (Department of Life Science, Pohang University of Science and Technology, Pohang, Korea). PLCβ3 isoforms in pcDNA3.1 were constructed from the cDNA of each of the mutated PLCβ3 isoforms. 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 × 10⁶ 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% CO₂. 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 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’-CAGCGCAGTCGCCCTCTCAATA-3’ and reverse 5’-ACCGCATTGGGCGATCCTC-3’; and β₂-microglobulin, used as a reference for normalization, forward 5’-GGTCTCTGTGGCTCTGGCATTACG-3’ and reverse 5’-GAGTACGCTGGGATGCTCCA-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 β₂-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 detection system (Bio-Rad). 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 pre-cleared 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

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**TABLE 1**

| Gene   | siRNA sequence (3’ linker, dTdT) | GenBank accession No. |
|--------|---------------------------------|-----------------------|
| Akt1   | GACAACCGCACAUCACGACU            | NM_00104431           |
| ERK1   | CAGAUCUUCUUAACGCAUCU            | NM_002745             |
| p38    | CUGUUGACUGGAGAACAAU             | NM_001315             |
| RSK1   | CUGUAGACUACCUCUCU               | NM_00106665           |
| CREB   | UCAGAGGAGCUCUUCCUACA            | NM_0054379            |
| c-Ets1 | GUGACUCUCAAAUAGGACA             | NM_005238             |
| Control| CUACGCCACCACAUUUGI              |                      |

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*Note:* Relative quantity of MUC5AC mRNA was obtained using the comparative cycle threshold method and was normalized using β₂-microglobulin as an endogenous control.

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**Gene siRNA sequences for indicated target genes**
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 EC50 value of 9.4 ± 0.15 μM (Fig. 1A), 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. 1B). 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.

PLC3 and Akt Are Essential for ATP-induced MUC5AC Gene Expression—To determine which molecules are involved in the downstream signaling of Gaq in ATP-induced MUC5AC gene expression, we investigated phospholipase C (PLC) β3 (Fig. 2A). The phosphorylation of PLC3 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 PLC3 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 PLC3 plays an important role in ATP-induced MUC5AC gene expression, each of the last four amino acid residues of PLC3 (L1233NTQL, L1234COOH) was mutated to Ala. When treated with ATP, cells transfected with the construct expressing wild-type PLC3 showed an increase in MUC5AC gene expression, whereas the cells transfected with the construct expressing the dominant-negative mutant PLC3 NTQA (L1234A), but not PLC3 ATQL (N1231A), NAQL (T1232A), or NTAL (Q1233A), showed a dramatic suppression of MUC5AC gene expression (Fig. 2B). This result indicates that the Leu residue of the PDZ domain in PLC3 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 (NTQL1234) or dominant-negative PLCβ3 (ATQL, NAQL, NTAL, NTQA) 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.
some target signaling molecule(s) to induce MUC5AC gene expression after ATP treatment. In addition, phosphorylation of PLC\(\beta\)3 and Akt were suppressed when cells were transfected with the PLC\(\beta\)3 NTQA dominant-negative construct (Fig. 2C). Even though many reports have shown that PLC\(\beta\)3 regulates PKC activation, it appears that PKC is not involved in ATP-induced signaling (Fig. 2D). 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 PLC\(\beta\)3 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 PLC\(\beta\)3. 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. 2F), and MUC5AC gene expression was significantly suppressed by siRNA-Akt. These results show that PLC\(\beta\)3 and Akt appear to function together in a pathway mediating ATP-induced MUC5AC gene expression and Akt functions downstream of PLC\(\beta\)3 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. 3A), 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. 3B). We also examined the transcript level of MUC5AC by performing real time PCR analysis (Fig. 3C). 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.
CREB and c-Ets1 Interact to Regulate MUC5AC Gene Expression

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. 4A, 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. 4B), 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. 4C). 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. 5A). 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. 5B). 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
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. 6A). 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. 6B). 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. 6C). Interestingly, siRNA-CREB suppressed the phosphorylation of c-Ets1, whereas siRNA-CREB suppressed the phosphorylation of 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...
CREB and c-Ets1 Interact to Regulate MUC5AC Gene Expression

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. 6D, 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. 6E). 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. 6F), 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. 6G, 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 cathepsin 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 Ca2+ 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 Goq 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 (PI3K) or an upstream site in the Akt pathway may be critical for inducing Akt activation in astrocytoma cells (26). 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. 4B). 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-PLCβ antibody was used as a negative control. Distilled water was used as a solvent of ATP as a vehicle control. These figures are representative of three independent experiments.
CREB and c-Ets1 Interact to Regulate MUC5AC Gene Expression

exposure of ATP (Fig. 5B). 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 over the role(s) of the Ets factor, because Ets-1 has dual functions to regulate cellular phenomena as follows: Ca2+-dependent phosphorylation of Ets1 results in the loss of DNA binding activity in murine T cell hybridoma Hdt11.2 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. 6F), 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. 6C, 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.

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