Protein Kinase A Activation of the Surfactant Protein B Gene Is Mediated by Phosphorylation of Thyroid Transcription Factor 1*

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Thyroid transcription factor 1 (TTF-1) is a homeodomain-containing nuclear transcription factor expressed in epithelial cells of the lung and thyroid. TTF-1 binds to and activates the transcription of genes expressed selectively in the respiratory epithelium including pulmonary surfactant A, B, C and Clara cell secretory protein. Transfection with a plasmid encoding the cyclic AMP-dependent protein kinase (protein kinase A; PKA) catalytic subunit, Cat-β, stimulated the phosphorylation of a TTF-1-flag fusion protein 6–7-fold in H441 pulmonary adenocarcinoma cells. Recombinant TTF-1 was phosphorylated by purified PKA catalytic subunit in the presence of [γ-32P]ATP. PKA catalytic subunit family members, Cat-α and Cat-β, markedly enhanced the transcriptional activation of surfactant B gene promoters by TTF-1 in vitro. Peptide mapping was used to identify a PKA phosphorylation site at the NH2-terminus of TTF-1. A 17-amino acid synthetic peptide comprising this site completely inhibited the PKA-dependent phosphorylation of TTF-1 in vitro. A substitution mutation of TTF-1 (Thr9 → Ala) abolished phosphorylation by PKA and reduced transactivation of the surfactant B gene promoter. Transfection with a plasmid encoding the cAMP regulatory element binding factor inhibited transcriptional activity of the surfactant protein B gene promoter. Phosphorylation of TTF-1 mediates PKA-dependent activation of surfactant protein B gene transcription.

Pulmonary morphogenesis begins with the evagination of the foregut endoderm into the splanchnic mesenchyme. Thereafter, branching morphogenesis and differentiation of respiratory epithelial cells result in the generation of the conducting airways and alveolar gas exchange areas critical to perinatal adaptation to air breathing. Respiratory function depends upon surfactant lipids and proteins that are secreted into the alveolus by type II epithelial cells, reducing surface tension at the air/liquid interface and keeping the lung from collapsing at end-expiration. Various humoral factors influence the differentiation of respiratory epithelial cells and the production of surfactant proteins and lipids. Surfactant proteins A, B, and C (SP-A, SP-B, and SP-C, respectively)† play critical roles in the organization and function of surfactant phospholipids and are expressed in a highly tissue-specific manner, their expression increasing markedly during the latter part of gestation (for review, see Ref. 1). SP-B, a 79-amino acid amphipathic peptide, plays an important role in the formation of lamellar bodies and tubular myelin and is critical to lung function after birth (2). Mice and humans with genetic defects in the SP-B gene succumb from respiratory failure in the immediate postnatal period (3, 4). In the fetal lung, SP-B synthesis increases markedly during late gestation and is induced by glucocorticoids and cAMP (5, 6).

The specificity of surfactant protein gene expression in respiratory epithelial cells is mediated at least in part by the binding of thyroid transcription factor 1 (TTF-1) to cis-acting elements in the 5′-flanking region of its target genes (7, 9). Transcription of SP-A (8), SP-B (7, 9), SP-C (10), and Clara cell secretory protein (CCSP; 7) is markedly activated by TTF-1. TTF-1 is a homeodomain-containing phosphoprotein of the Nkx family and is expressed in the developing brain, thyroid, and lung (11). TTF-1 also plays a critical role in pulmonary morphogenesis. Gene-targeted deletion of the mouse TTF-1 gene (titf1) caused severe pulmonary hypoplasia (12). TTF-1 also plays a role in gene expression in the thyroid gland, activating thyroperoxidase and thyroglobulin gene transcription (23, 24, 32). Because of the role of TTF-1 in surfactant protein gene expression and previous studies supporting the role of cyclic AMP in lung epithelial differentiation and function, the present study was designed to assess the potential interactions between cAMP-dependent pathways and the activity of TTF-1.

The action of cAMP is initiated by binding of hormones or other signaling molecules to cell surface receptors stimulating the activity of adenylyl cyclase in the plasma membrane, activating protein kinases that phosphorylate regulatory proteins, including nuclear transcription factors (13–15). A number of intracellular signaling pathways have been elucidated including protein kinase A (PKA), protein kinase C (PKC), and Ras/Raf/MEK/ERK, Rac/MEK/SEK/JNK, JAK/STAT (for review, see Ref. 16). The potential role of these intracellular regulatory cascades in respiratory epithelial cell gene expression has not been discerned at present.

The actions of PKA on gene transcription are mediated by phosphorylation of various transcription factors including CREBs, CREM, and other CRE binding proteins (ATFs) that influence gene transcription (for review, see Refs. 14 and 17). Although cAMP is known to stimulate respiratory epithelial cell differentiation and surfactant protein expression (6), the

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† The abbreviations used are: SP-A, SP-B, and SP-C, surfactant proteins A, B, and C, respectively; TTF-1, thyroid transcription factor 1; CCSP, Clara cell secretory protein; PKA, protein kinase A; PKC, protein kinase C; CRE, cAMP-responsive element; CREB, CRE-binding factor; CREM, CRE modulator; CBP, CREB-binding protein; ATF, activation transcription factor; Cat, catalytic subunit of PKA; HNF-3, hepatocyte nuclear factor 3; wt, wild type.
intracellular pathways mediating the effects of cAMP or gene transcription in the lung are unknown. In the present work, the PKA catalytic subunits Cat-α and Cat-β, but not CREB, strongly stimulated the human SP-B promoter in lung epithelial cells. Cat-β phosphorylated TTF-1 and synergistically stimulated human SP-B promoter when cotransfected with TTF-1, into H441 pulmonary adenocarcinoma and HeLa cells.

MATERIALS AND METHODS

*Plasmids—* Human SP-B gene promoter-luciferase reporter construct, SP-B-500 and SP-B-500-b, were generated as described previously (18). PKA catalytic subunit expression vector Cat-α, Cat-β and inactive form Cat-βmut were obtained from Dr. R. A. Maurer, Oregon Health Sciences Institute (19, 20). Re-TTF-1 expression vector was obtained from Dr. R. Di Lauro, Stazione Zoologica Anton Dohrn, Italy. CRE expression vector was obtained from Dr. M. R. Montminy, Salk Institute, La Jolla, CA. pCR3 vector was purchased from Invitrogen (San Diego). Re-HNF-3α, Re-HNF-3β and Re-HFPH-8 expression vectors were obtained from Dr. R. H. Costa from University of Illinois at Chicago.

The wild type TTF-1-flag expression construct (TTF-1 wt) was made as follows. Two primers were synthesized and used for polymerase chain reaction amplification using TTF-1 expression plasmid from Dr. R. Di Lauro as a template. The upstream primer (5-GGCCACCATGTC-GAGTCCAAAGCACACGACT-3) contains a Kozak sequence for efficient translation and 21-mer matching the 5’ end of TTF-1 cDNA sequence. The downstream primer (3’ACGAAATACCGCTTGAC-CCTGATGCTCTCCTGACTATCTATT5’) contains a 21-mer matching the 3’ end of TTF-1 cDNA sequence and a in-frame flag sequence (underlined sequence) before two stop codons. A mutant TTF-1-flag construct consists of a Thr9→Ala substitution; TTF-1 Thr9→Ala was made essentially the same except the upstream primer has the sequence 5’-GCCACCATGTC-GAGTCCAAAGCACACGACT-3’ (contains a Kozak sequence for 21-mer matching the 3’ end of TTF-1 cDNA sequence and a in-frame flag sequence (underlined sequence) before two stop codons). A mutant TTF-1-flag construct consists of a Thr9→Ala substitution; TTF-1 Thr9→Ala was made essentially the same except the upstream primer has the sequence 5’-GCCACCATGTC-GAGTCCAAAGCACACGACT-3’ (contains a Kozak sequence for efficient translation and 21-mer matching the 5’ end of TTF-1 cDNA sequence). The polymerase chain reaction product of TTF-1 with the Kozak and flag sequences was subcloned directly into the pCR3 TA cloning vector (Invitrogen) to generate pCR3/TTF-1-flag expression construct. The sequences was subcloned directly into the pCR3 TA cloning vector (Invitrogen) to generate pCR3/TTF-1-flag expression construct. The orientation and DNA sequence of the constructs were confirmed by DNA sequencing.

TTF-1-his-tag fusion protein construct for bacterial expression was made previously (21). The TTF-1 cDNA was directionally cloned into the pET 21b bacterial expression vector (pET System, Novagen Inc., Madison, WI). BL21(DE3) *Escherichia coli* was transformed, and expression of recombinant TTF-1 was induced with the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 2 mM. Nicotinamide adenine dinucleotide phosphate (NADP+) and nitroblue tetrazolium were added to the growing culture to enhance the bacterial growth. The cells were harvested and resuspended in 20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 2 mM MgCl2, 10% glycerol, 100 μM EDTA, and 100 μM valine. The cells were centrifuged at 3000 rpm for 30 min, and the supernatant was discarded. The protein A/G agarose (Santa Cruz Biotechnology) was added and the supernatant was incubated overnight with 10 μl of flag M2 monoclonal antibody (Kodak) or 5 μl of TTF-1 monoclonal antibody at 4 °C on a rotator. The next day, 20 μl of protein A/G plus-agarose was added and the incubation continued for 90 min. The supernatant was discarded and agarose complex centrifuged, washed three times with cell lysis buffer, and washed once with buffer containing 150 mM NaCl, 50 mM Tris, pH 7.5, and 5 mM EDTA. Samples were boiled in sample loading buffer, centrifuged, and the supernatant proteins separated by sodium dodecyl sulfate-gel electrophoresis as described by Laemmli (22). The intensity of protein phosphorylation was visualized after exposure of the dried gels to x-ray film and quantitated by PhosphorImaging.

Western blot analysis of nuclear protein extracts from TTF-1 wt and TTF-1 (Thr9→Ala) transfected H441 cells was performed using the Phast Gel system (Pharmacia Biotech Inc.).

**RESULTS**

The Catalytic Subunit of PKA Activated the Human SP-B Promoter—Transcription of the human SP-B gene is conferred by cis-acting elements located −500 to +41 from the start of transcription and is dependent upon the binding of TTF-1 to distinct sites within this region (7, 9). To assess the role of cAMP-dependent protein kinases on SP-B gene transcription, plasmid expression vectors expressing two forms of PKA catalytic subunit, Cat-α and Cat-β, were cotransfected into H441 cells with the SP-B promoter fragment, SP-B-500-luciferase. Cat-α and Cat-β stimulated SP-B gene transcription (Fig. 1A). Activation of SP-B-500-luciferase was dose-dependent, and the inactive form of PKA catalytic subunit, Cat-βmut, had no effect on the activity of SP-B-500-luciferase. Cat-β was consistently more active than Cat-α. The Cat-β construct was therefore utilized for subsequent studies.

To define further the region of the SP-B promoter which was responsive to PKA, SP-B-218-luciferase (containing −218 to +41) was also tested. Activity of SP-B-218-luciferase was markedly stimulated by PKA Cat-β (Fig. 1B), supporting the concept that DNA elements responding to Cat-β resided within −218 to +41 of the human SP-B gene, a region containing cis-acting elements activated by TTF-1 (7). When the mutant SP-B-218 promoter (SP-B-218 TT, Ref. 7) lacking TTF-1 binding activity was used, no stimulation was observed by PKA Cat-β (Fig. 1B). DNA homology search revealed no CRE consensus elements within this region of the SP-B promoter, suggesting that the stimulatory effects of PKA Cat-β on SP-B-218-luciferase were not mediated by direct activation of CRE elements in the SP-B gene. Importantly, direct cotransfection of H441 cells with the expression plasmid RSV-CREB inhibited transcription of SP-B-500-luciferase in H441 cells (data not shown).

**TTF-1 and HNF-3 Transactivated the Human SP-B Promoter—**To further identify transcription factors regulating the activity of the SP-B promoter, the effects of TTF-1, HNF-3α, HNF-3β, and HFPH-8 expression vectors on the activity of SP-B-218-luciferase were assessed in H441 cells (Fig. 2). Cotransfection with a vector expressing TTF-1 consistently activated SP-B-218-luciferase (6–7-fold), whereas HNF-3-family members were less active. Cotransfection of H441 cells. Stimulation of SP-B-218-luciferase by TTF-1 (−6 fold) was similar to that observed after transfection with Cat-β (5-fold) in H441 cells. Addition of 8-bromo-cAMP to H441 cells increased SP-B-500-luciferase activity approximately 2-fold, 24 h after treatment (data not shown).

PKA Catalytic Subunit-stimulated Phosphorylation of TTF-1 in H441 cells—To assess whether TTF-1 was phosphorylated by PKA catalytic subunit, a TTF-1-flag construct (pCR3/TTF-1-flag) was generated. A flag sequence (Asp-Tyr-Lys-Asp-Asp-
Asp-Asp-Lys) was inserted into the COOH terminus of the rat TTF-1 polypeptide producing a TTF-1 fusion peptide that was used to immunoprecipitate and distinguish endogenous TTF-1 from the flag-TTF-1 produced by the pCR3/TTF-1-flag construct. pCR3/TTF-1-flag transactivated SP-B-500-luciferase in H441 cells, demonstrating that the TTF-1-flag fusion peptide retained its biological activity (data not shown).

To test whether PKA increased the phosphorylation of TTF-1, pCR3/TTF-1-flag was cotransfected with either Cat-β or Cat-βm into H441 cells (Fig. 3). After radiolabeling with 32P, TTF-1 from the cell lysates was immunoprecipitated with the flag antibody. Cat-β markedly increased the phosphorylation of TTF-1-flag, the protein migrating in approximately the same position as endogenous phosphorylated TTF-1. Cat-β enhanced TTF-1-flag phosphorylation approximately 6–7-fold compared with Cat-βm in H441 cells. Phospho-TTF-1-flag consisted of two bands (M r = 40,000–43,000), supporting the concept that TTF-1 was phosphorylated at multiple sites or that multiple forms of TTF-1 were present.

**PKA Catalytic Subunit-phosphorylated TTF-1-his-tag in**
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**Fig. 4.** Phosphorylation of TTF-1-his-tag protein. Recombinant TTF-1-his-tag protein was purified by Ni-column chromatography. Purified TTF-1-his-tag fusion protein (1 μg) and TTF-1 homeodomain (HD) polypeptide (1 μg) were incubated with the purified PKA catalytic subunit and [γ-32P]ATP for 1 h at 30 °C and separated on a 10% polyacrylamide gel. The arrow indicates the phosphorylated TTF-1.

**Fig. 5.** Identification of the PKA phosphorylation site on TTF-1. Panel A, peptide mapping of the PKA phosphorylation sites on TTF-1. Four synthetic peptides (30 μg), comprising TTF-1 phosphorylation sites previously identified (23), and TTF-1-his-tag (positive control) were incubated with the purified PKA catalytic subunit and [γ-32P]ATP for 45 min at 30 °C. The reaction mixtures were spotted onto the phosphocellulose disc, washed, and counted by scintillation counter. Peptide 1 and TTF-1-his-tag were phosphorylated by PKA catalytic subunit. For each peptide or protein, radioactivity of phosphorylation in the absence of PKA catalytic subunit is set as 1. Values are the mean of two independent assays. Peptide 1 comprises the amino acid sequence from 1 to 17 of TTF-1; peptide 2, from 13 to 29; peptide 3, from 322 to 342; peptide 4, from 248 to 260. Panel B, inhibition of TTF-1-his-tag phosphorylation by TTF-1 synthetic peptides. Purified TTF-1-his-tag protein (1 μg) was incubated with the purified PKA catalytic subunit and γ-32P in the absence (lane c) or presence of synthetic peptides (lanes d, e, f, and g, respectively) for 45 min at 30 °C and separated on a 10% polyacrylamide gel. Peptide 1 (lane d) inhibited the phosphorylation of TTF-1-his-tag. Lane a contains the negative control for TTF-1-his-tag fusion protein, lane b contains a control lacking the PKA catalytic subunit. The arrow indicates the phosphorylated TTF-1.

**Vitro**—Bacterial expressed TTF-1-his-tag protein was purified by Ni-column chromatography. Purified TTF-1-his-tag fusion protein and TTF-1 homeodomain polypeptide were incubated with the purified PKA catalytic subunit in the presence of [γ-32P]ATP and separated on a 10% polyacrylamide gel. As shown in Fig. 4, PKA catalytic subunit directly phosphorylated the TTF-1 fusion protein (Fig. 4, lane c), but not the TTF-1 homeodomain polypeptide, indicating that the PKA phosphorylation site resided outside the homeodomain of TTF-1. To identify the site of PKA phosphorylation, a set of synthetic peptides comprising TTF-1 phosphorylation sites was generated.

**Identification of TTF-1 Peptide Containing the PKA Phosphorylation Site**—Di Lauro and colleagues identified several phosphorylation sites on TTF-1 (23). Four peptides were synthesized covering these phosphorylation sites. The peptides were incubated with the purified PKA catalytic subunit in the absence of [γ-32P]ATP. Only peptide 1 (residues 1–17) was phosphorylated by the PKA catalytic subunit (Fig. 5A). In competition experiments, peptide 1 abolished TTF-1-his-tag protein phosphorylation by PKA (Fig. 5B, lane d). These results demonstrated that the PKA phosphorylation site was located near the NH₂ terminus of TTF-1 in a region associated with transactivation activity (24).

**TTF-1 (Thr⁹ → Ala) Mutation Blocks Phosphorylation by PKA but Not PKC**—Peptide 1 contains the sequence Arg-His-Thr-Thr-Pro consistent with a consensus sequence for phosphorylation by PKA (14). A PKC consensus sequence Ser-Pro-Arg-His that shares Arg-His with the PKA site, is located immediately proximal to the NH₂ terminus of the putative PKA site (Fig. 6A). A mutant peptide 1 (peptide 1m1), in which Thr⁹ was changed to Ala (Arg-His-Thr-Thr-Pro → Arg-His-Thr-Ala-Pro), was synthesized and tested as substrate for PKA and PKC in vitro. As shown in Fig. 6B, the phosphorylation of mutant peptide 1m1 by PKA was completely abolished, indicating that the mutant peptide 1m1 by PKC was fully retained.

**TTF-1(Thr³ → Ala) Reduced Transactivation of the SP-B Gene Promoter**—A mutant form of TTF-1 (Thr³ → Ala) was generated by mutation of the TTF-1 cDNA (see “Materials and Methods”). Whereas TTF-1 wt stimulated the activation of the SP-B promoter (SP-B-218), TTF-1 (Thr³ → Ala) was considerably less active in stimulating transcription of SP-B-218-luciferase (Fig. 7). Differences in the activity of TTF-1 wt and TTF-1 (Thr³ → Ala) were not related to differences in the level of TTF-1 peptides produced by the constructs. Western blot analysis of TTF-1 wt and TTF-1 (Thr³ → Ala)-transfected H441 cells demonstrated equal amounts of TTF-1 fusion proteins (data not shown). Immunofluorescent staining by anti-flag monoclonal antibody showed that TTF-1 wt and TTF-1 (Thr³ → Ala) were nuclear localized and strongly expressed in the transfected cells (data not shown).
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FIG. 6. Mutation of Thr\(^9\) to Ala inhibits phosphorylation of TTF-1. Panel A, PKA and PKC phosphorylation sites within peptide 1. Panel B, mutation (Thr\(^9\) → Ala) in peptide 1 blocked phosphorylation by PKA but not by PKC. 30 μg of peptide 1 and mutant peptide 1 (Thr\(^9\) → Ala) were incubated with purified PKA catalytic subunit or PKC and [γ\(^-\)\(^32\)P]ATP for 45 min at 30 °C. The reaction mixtures were spotted on phosphocellulose discs, washed, and counted. Peptide 1 was phosphorylated by both PKA catalytic subunit and PKC. On the other hand, mutant peptide 1 (peptide 1m1) was phosphorylated by PKC but not by PKA. For both peptides, phosphorylation in the absence of PKA catalytic subunit is set as 1. Values are the mean of two independent assays.

SP-B-218 promoter, the phosphorylation mutant TTF-1 (Thr\(^9\) → Ala) was transfected into H441 cells and failed to mediate Cat-β-dependent stimulation of SP-B-218-luciferase in H441 cells (Table I).

**TABLE I**

| Plasmids         | Luciferase activity ± S.D. |
|------------------|---------------------------|
| PKA wt + PKA Cat-β   | 4,880 ± 316               |
| PKA wt + PKA Cat-β   | 12,040 ± 1,550            |
| TTF-1 (Thr\(^9\) → Ala) + PKA Cat-β | 2,320 ± 351                |
| TTF-1 (Thr\(^9\) → Ala) + PKA Cat-β | 3,100 ± 178                |

**FIG. 7.** TTF-1 (Thr\(^9\) → Ala) inhibits the activity of the SP-B-218-luciferase reporter. H441 cells (4 × 10⁵) were transfected with plasmid DNA containing 3.3 μg of SP-B-218-luciferase reporter vector, varying amounts of TTF-1 wt or TTF-1 (Thr\(^9\) → Ala) expression vectors (0, 1.6, 3.3, and 5.0 μg, respectively), and varying amounts of carrier DNA to maintain constant DNA concentration. The activity of SP-B-218-luciferase in the absence of the TTF-1 expression vector was defined as 1. Values are mean ± S.D., n = 3.

**DISCUSSION**

Respiratory adaptation at birth depends upon the regulated synthesis and secretion of surfactant lipids and proteins into the alveolar spaces, reducing surface tension at the air/liquid interface. The synthesis of surfactant lipids and proteins is developmentally regulated, increasing during advancing gestation and stimulated by a variety of humoral factors including glucocorticoids and cAMP. The present findings demonstrate that PKA activated both TTF-1 phosphorylation and TTF-1-dependent activation of SP-B promoter activity, suggesting that the effects of cAMP on surfactant protein B gene expression may be mediated, at least in part, by the modulation of the activity of TTF-1 on the SP-B transcription, rather than by binding and activating CRE.

TTF-1 is expressed throughout the conducting and peripheral respiratory tract during lung development (11, 25). In human lung, after birth, TTF-1 is detected in nuclei of subsets of epithelial cells, both in the conducting airway and in type II epithelial cells (25, 26). In the lung, TTF-1 expression overlaps with that of SP-A, SP-B, SP-C, and CCSP, but its distribution is more extensive than that of the surfactant proteins or CCSP (27–29). Although TTF-1 strongly influences the transcription of each of these genes, each has a distinct temporal and spatial pattern of expression, supporting the concept that the activity of TTF-1 may be further modified to confer the distinct regulatory features typical of each gene. The marked increase in surfactant protein synthesis occurring in late gestation is not directly related to changes in the level of TTF-1 mRNA (30), providing a strong inference that the activity of TTF-1 on its target genes may be further modulated. The present work supports the hypothesis that the stimulatory effects of cyclic AMP on surfactant homeostasis may be influenced, at least in part, by phosphorylation of TTF-1, which, in turn, enhances its activity on SP-B gene transcription.

Effects of cAMP on gene transcription are mediated by the activation of PKAs; subsequent protein phosphorylation may alter the activity of regulatory proteins including nuclear transcription proteins. A variety of nuclear transcription proteins are targets of PKA. Some bind to CREs that are present in target genes. Many genes that are regulated by PKA contain the CRE consensus sequence TGACGTC (17). We were unable to identify CRE sequences fitting this consensus sequence in the 5′-flanking regions of mouse SP-A and human or mouse SP-B and SP-C genes. Likewise, expression of recombinant CREB failed to activate SP-B gene transcription from the SP-B-500-luciferase construct in H441 cells. SP-A gene expression is also enhanced by cAMP and is not mediated by CREB/ATF family members (31). In the present work, SP-B transcription was markedly stimulated by cotransfection with TTF-1, and this activity was further induced by cotransfection with Cat-α and -β, suggesting that the activation by the catalytic subunit of PKA may be mediated by enhancing the activity of TTF-1 on the SP-B promoter.

TTF-1 phosphorylation was markedly stimulated by cotransfection with Cat-β, but not by the inactive form, Cat-βm. These findings are consistent with observations in thyroid carcinoma...
influenced by hormonal factors that stimulate lung cell differen-
tors and the activity of adenylate cyclase in the lung increase
dramatically during late gestation (33, 34) and are further
influenced by hormonal factors that stimulate lung cell differen-
tiation (35). In late gestation, the numbers of β-adrenergic recep-
tors in the lung increase in association with the increased
surfactant production and secretion required for perinatal respir-
tory adaptation. The present findings support the concept
that the effects of cAMP on distal respiratory epithelial function
may be mediated, at least in part, by the activity of TTF-1,
which modulates surfactant protein gene transcription. Whether
other effects of cAMP on respiratory epithelial cell function in the
perinatal lung are mediated by cAMP-dependent
activation of TTF-1 remains to be clarified.

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