Role of CBP/p300 and SRC-1 in Transcriptional Regulation of the Pulmonary Surfactant Protein-A (SP-A) Gene by Thyroid Transcription Factor-1 (TTF-1)*

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Surfactant protein-A (SP-A) gene expression is developmentally regulated in fetal lung type II cells and is enhanced by cAMP. cAMP stimulation of SP-A gene expression is mediated by protein kinase A (PKA) phosphorylation of thyroid transcription factor 1 (TTF-1), expressed selectively in developing lung epithelium. In this study, we analyzed roles of CREB-binding protein (CBP) and steroid receptor coactivator-1 (SRC-1) in TTF-1 regulation of SP-A expression. Upon differentiation of human fetal lung in culture, nuclear localization of CBP, SRC-1, and TTF-1 increased in ductular epithelium in association with type II cell differentiation and induction of SP-A expression. In transient transfections, CBP and SRC-1 acted synergestically with TTF-1 to increase SP-A promoter activity. Overexpression of PKA catalytic subunit enhanced hSP-A promoter activation by SRC-1 plus TTF-1. Adenoviral E1A overexpression reduced TTF-1 ± SRC-1 induction of SP-A promoter activity, suggesting a role of endogenous CBP/p300. TTF-1 interacted with SRC-1 and CBP in vitro. SRC-1 immuno-depletion from type II cell nuclear extracts reduced binding to the TTF-1 binding element upstream of SP-A gene. In cultured type II cells, cAMP increased TTF-1 acetylation. This suggests that cAMP-mediated TTF-1 phosphorylation facilitates interaction with CBP and SRC-1, resulting in its hyperacetylation, further enhancing TTF-1 DNA-binding and transcriptional activity.

Pulmonary surfactant, a developmentally regulated phospholipid-rich lipoprotein synthesized exclusively in lung type II cells, reduces surface tension, thereby preventing alveolar collapse upon exhalation of air. There are four lung-specific surfactant-associated proteins: SP-A, SP-B, SP-C, and SP-D, which serve a number of different roles, including enhancement of surface-active properties of surfactant glycerophospholipids, surfactant phospholipid reutilization, and immune defense within the alveolus (1). SP-A, the major surfactant protein, is a C-type lectin that activates macrophages in the lung alveolus and plays an important role in immune defense (2). SP-A is synthesized primarily by type II pneumocytes (3) and is developmentally regulated in fetal lung in concert with surfactant glycerophospholipid synthesis (4, 5). Transcription of the SP-A gene is initiated in fetal lung after ~70% of gestation is completed and reaches maximum levels just prior to birth (6). In humans and baboons, SP-A is encoded by two highly similar genes, SP-A1 and SP-A2 (7, 8), whereas, in rabbits (9), rats (10) and mice (11), SP-A is encoded by a single-copy gene.

cAMP has major stimulatory effects on SP-A expression in human (12), baboon (13), and rabbit (4, 5) fetal lung in culture. The human (h)SP-A2 gene is far more responsive to the stimulatory effects of cAMP than is hSP-A1 (14, 15). In type II cell transfection studies, we observed that basal and cAMP-induced hSP-A2 promoter activity is critically dependent upon four regulatory elements within the proximal SP-A 5'-flanking region. These elements, which are highly conserved in the 5'-flanking regions of the SP-A genes of various species (16), include a putative nuclear receptor binding site (17, 18), a GT-box that binds Sp1 and related Krüppel factors (19), an E-box that binds USFs 1 and 2 (20) and 2 and a binding site (TBE) for thyroid transcription factor-1 (TTF-1) (21, 22). Each of these elements is crucial for basal and cAMP-induced levels of SP-A gene expression.

TTF-1, also referred to as thyroid enhancer-binding protein (T/ebp) and Nkx2.1, is a homeodomain-containing transcription factor that was originally reported to be involved in regulation of a number of thyroid-specific genes (23–25). TTF-1 is expressed only in developing lung, thyroid, and diencephalon (26, 27). Expression in thyroid and lung is evident from the earliest stages of development (26). The finding that the TTF-1 null mouse lacks thyroid and lung parenchyma, as well as anterior pituitary, indicates that TTF-1 serves a critical role in morphogenesis of these tissues (28). TTF-1 also has been found to activate transcription of SP-A, SP-B, SP-C, and Clara cell-specific protein (CC10) genes, which are expressed exclusively in lung epithelium (21, 29–32).

We have identified and characterized three TTF-1 binding elements (TBEs) within the 5'-flanking regions of baboon (b) and human (h) SP-A1 and SP-A2 genes (22). The TBE core consensus sequence located at −178 bp (TBE1) was found to be shift assay; hGH, human growth hormone; Br, cAMP, dibutyryl cAMP; GST, glutathione S-transferase.

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most highly conserved and functionally critical for cAMP induction of SP-A promoter activity (21). We also observed that cAMP acting through a CAMP-dependent protein kinase (PKA) increased TTF-1 phosphorylation, TBE1 binding, and transcriptional activity in lung type II cells (21).

As mentioned above, TTF-1 is expressed in pulmonary epithelium from the earliest stages of lung morphogenesis (26). By contrast, SP-A gene transcription is initiated in association with type II cell differentiation, only after ~70% of gestation is completed (5, 6). In light of our previous findings that cAMP/PKA increases TTF-1 DNA-binding and transcriptional activity, we postulate that developmental changes in TTF-1 post-translational modification may facilitate its interaction with coactivators to mediate temporal regulation of SP-A gene transcription. Coactivators enhance transcriptional activation through interactions with transcription factors and components of basal transcription complex. Although they do not bind to DNA directly, such protein-protein interactions stabilize DNA binding and assembly of the basal transcription complex. Some coactivators contain intrinsic histone acetyltransferase (HAT) activity, which facilitates opening of chromatin structure, allowing increased accessibility of gene regulatory elements to transcription factors (reviewed in Refs. 33, 34).

CREB-binding protein (CBP) and its structural homologue p300 were initially characterized as coactivators required for efficient activation of CAMP-regulated promoters (35, 36). CBP/p300 also have been implicated in cell growth, differentiation, and development (36, 37). Steroid receptor coactivator-1 (SRC-1), the first member of p160 coactivator family to be characterized (38), can form a complex with CBP/p300 and synergistically activate transcription of target genes (39, 40). Both CBP/p300 and SRC-1 possess HAT activities.

In consideration of the crucial role of TTF-1 in lung development and in cAMP activation of SP-A gene expression, it was of interest to further define possible mechanisms that modulate TTF-1 activity. In the present study, we analyzed the roles of CBP and SRC-1 in TTF-1 induction of SP-A promoter activity. We found that CBP and SRC-1 acted synergistically with TTF-1 to increase SP-A2 promoter activity, and that adenoviral E1A, a specific inhibitor of CBP/p300, blocked the stimulatory effects of TTF-1 and SRC-1, suggesting a role of endogenous CBP/p300. The importance of CBP and SRC-1 in TTF-1 regulation of SP-A expression was further emphasized by the novel finding that nuclear localization of CBP, SRC-1 and TTF-1 increased with differentiation and induction of SP-A expression. TTF-1 also was found to physically interact with SRC-1 and CBP in vitro. In electrophoretic mobility shift assays (EMSA), we observed that immunodepletion of SRC-1 from type II cell nuclear extracts resulted in decreased binding to the TBE, suggesting that the TTF-1-SRC-1 interaction increases TBE-binding activity. Our intriguing finding that cAMP increased the rate of TTF-1 acetylation in cultured type II cells further suggests that activation of cAMP signaling pathways may facilitate TTF-1 association with coactivators with intrinsic HAT activity. Thus, in turn, may contribute to increased TTF-1 DNA-binding activity and result in an opening of chromatin structure, stabilization of the preinitiation complex, and activation of transcription.

MATERIALS AND METHODS

Plasmids—An expression vector (pCMV5/TTF-1) containing the full-length cDNA encoding baboon TTF-1 (22) was constructed as described previously (21). An expression vector for PKA catalytic subunit, RSV/PKA-cat-β, was kindly provided by Dr. Richard A. Maurer (Oregon Health Science University). A human growth hormone (hGH) reporter plasmid ((TBE)5-SP-A-hGH) containing a concatamer of three DNA repeats of bSP-A2 gene 5′-flanking sequence between –186 and –186 bp, which contained TBE1 (underlined, 5′-GTCCTCCCTCAGGCGTCTA-3′), 50 bp of 5′-flanking sequence, and 40 bp of the first exon of bSP-A2, was constructed as described previously (21). Reporter plasmids containing 255 bp of 5′-flanking sequence and 40 bp of the first exon of bSP-A2 linked to hGH (bSP-A2-255-hGH) and bSP-A2-255-M1:hGH, containing a mutation in TBE1, were constructed as described previously (21). SRC-1, was kindly provided by Drs. Carolyn Smith and Bert O’Malley ( Baylor College of Medicine). E1A and CBP expression vectors (pRC/RSV-cBP-HA.RK) were kindly provided by Dr. Marc Montminy (Salk Institute).

Isolation and Culture of Lung Type II Cells and Undifferentiated Epithelial Cells and Culture of Cell Lines—For isolation of type II cells, mouse or human fetal lung explants were obtained from organ cultures in serum-free Waymouth’s MB752/1 medium (Invitrogen) containing Bt2cAMP (Roche Molecular Biochemicals) for 3 days to promote type II cell differentiation (22). After culture, the tissues were digested with collagenase, and the isolated cells were treated with DEAE-dextran for enrichment of type II cells, as previously described (41). Undifferentiated epithelial cells were isolated from midgestation human fetal lung tissue prior to culture. The minced fetal lung tissues were digested with collagenase and treated with DEAE-dextran, as described for type II cell isolation. The enriched type II cells and undifferentiated epithelial cells were plated on an extracellular matrix prepared from Madin-Darby canine kidney cells and cultured at an air-liquid interface in serum-free Waymouth’s MB752/1 medium in the absence or presence of Bt2cAMP (41). A549 human lung adenocarcinoma cells (ATCC CCL 185) were cultured in Waymouth’s MB752/1 medium containing 10% (v/v) fetal bovine serum.

Transient Transfections—Before transfection, A549 cells were plated onto 35-mm dishes and grown to logarithmic phase at 50–80% confluence. After washing 3× with Hanks’ balanced salt solution (pH 7.4, Invitrogen), the cells were cotransfected with 2 μg of either bSP-A2-255-hGH, bSP-A2-255-M1:hGH, or (TBE)5-SP-A2-hGH reporter plasmids, expression vectors for TTF-1, PKA-catβ, SRC-1, CBP, and/or E1A, as well as the corresponding amounts of empty vectors, and 0.2 μg of RSV/β-Gal as a control for transfection efficiency. Each experimental condition was assayed in triplicate. Four micrograms of plasmid DNA for each transfection were incubated with Fugene6 (Roche Molecular Biochemicals) in Waymouth’s MB752/1 medium without serum, as instructed by the manufacturer, before adding to cells. The cells were incubated with the Fugene6/DNA mixture for 6–18 h at 37 °C before washing in Waymouth’s MB 752/1 medium. The cells were then incubated for 24 h, and the media were collected and assayed for hGH by radioimmunoassay using a hGH radioimmunoassay kit (Nichols Institute Diagnostics, CA). Variations in transfection efficiency were normalized by assay of β-galactosidase activity using a Galacto-Light kit (Tropix, MA), as indicated by the manufacturer.

GST Pull-down Assays—Full-length TTF-1 and various fragments, including the N-terminal region (amino acids 1–207), containing the N-terminal activation domain, the C-terminal region (amino acids 207–371), containing the C-terminal activation and inhibitory domains, and the homeodomain region (amino acids 148–227), were subcloned into pGEX-Fusion vectors (Amersham Biosciences, Inc.). The GST protein and GST fusion proteins containing various TTF-1 fragments were prepared by transforming the expression vectors into DH5α F’IQ (Invitrogen) bacteria and inducing expression with appropriate isopropl-1-thio-β-D-galactopyranoside concentrations. GST protein and GST fusion proteins linked to glutathione-agarose beads (Amersham Biosciences, Inc.) were combined with 30 μl of in vitro transcribed/translated [35S]methionine-labeled SRC-1 or CBP and incubated at 4 °C for 2 h. The beads were then washed 4× with wash buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM MgCl2, 1 mM diithiorethiol, 1 mM protease inhibitor mixture, Roche Molecular Biochemicals) at 4 °C. Bound proteins were eluted in SDS sample buffer and subjected to SDS-PAGE and autoradiography.

EMSA—Nuclear extracts from lung type II cells treated with Bt2cAMP for 3 days were prepared, as described previously (21, 42). Protein concentrations were measured using a modified Bradford assay (Bio-Rad). A double-stranded oligonucleotide, comprised of 186 to 167 bp upstream of the SP-A2 gene, containing TBE1 (underlined) (5′-GTCCTCCCTCAGGCGTCTA-3′; heretofore referred to as TBE probe), end-labeled with [γ-32P]ATP, was used as probe. Nuclear protein was precipitated with 0.5 μg of poly(dI-dC)-poly(dI-dC) (Amersham Biosciences, Inc.), as nonspecific competitor. EMSA was also carried out using type II cell nuclear extracts immunodepleted of CBP, TTF-1, or SRC-1. For immunodepletion, the nuclear extracts (~90 μg) were incubated at 4 °C for 1 h with either rabbit or goat nonimmune IgG (0.6 μg),
or with corresponding amounts of IgG for CBP, SRC-1, or TTF-1, followed by incubation at 4 °C for 1 h with protein A/G plus-agarose beads (Santa Cruz Biotechnology) and centrifugation to spin down the beads. Aliquots of the immunodepleted nuclear extracts were then incubated with radiolabeled TBE probe, as described above. For antibody-mediated supershift EMSA, the nuclear extracts were preincubated for 30 min with antibodies to CBP or SRC-1 (1 μg) at room temperature prior to incubation with radiolabeled TBE probe. Protein-DNA complexes were resolved on a 4% native polyacrylamide gel and visualized by autoradiography. Antibodies for CBP and SRC-1 were obtained from Santa Cruz Biotechnology; TTF-1 antisera was raised and prepared as previously described (21).

**Immunoblot Analysis**—Human fetal lung explants cultured for up to 3 days in serum-free medium, in the absence or presence of Bt2cAMP (1 mM), were homogenized in ice-cold phosphate-buffered saline containing protease inhibitor mixture (1 tablet/10 ml) (Roche Molecular Biochemicals). Proteins (30 μg) were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes as described previously (12). The membranes were then analyzed for SP-A using a specific antisem (12) and an enhanced chemiluminescence system (ECL) according to the manufacturer’s recommendations (Amersham Biosciences, Inc.).

**Immunocytochemistry**—Midgestation human fetal lung tissues collected before and after organ culture for 1–3 days in serum-free Waymouth’s MB752/1 in the absence or presence of Bt2cAMP (1 mM) were deparaffinized with xylene and hydrated in graded ethanol washes. Slides were blocked with DAKO serum-free protein block for 30 min at room temperature. Immunostaining was performed using a Vectastain Elite ABC kit, essentially according to the manufacturer’s instructions. Incubations with primary antibodies for CBP (Santa Cruz Biotechnologies), p300 (Santa Cruz), SRC-1 (Affinity Bioreagents), or TTF-1 (21) were performed at 4 °C overnight. Modifications to the manufacturer’s protocol included washing of sections for 10 min in 1% Triton X-100 (1%) after incubation with primary antibodies, and incubation of sections for 10 min in 0.5% H2O2 (peroxidase blocking) after incubation with the biotinylated secondary antibody, but before addition of the ABC reagent. The colorimetric reagent used was Vector Nova Red. The positive signal is reddish orange. No counter-staining was performed.

**In Vivo Acetylation Assay**—Epithelial cells isolated from midgestation human fetal lung, as described above, were cultured in serum-free Waymouth’s MB752/1 medium with or without Bt2cAMP for 3 days. Approximately 10^7 cells were metabolically labeled with [3H]sodium acetate (1 μCi/ml) for 2 h. Whole cell extracts were prepared in RIPA buffer and immunoprecipitated with TTF-1 antisem or pre-immune serum, as control (21). The immunoprecipitated proteins were resolved by 8% SDS-PAGE. Gels were fixed in 25% isopropanol/10% acetic acid prior to impregnation for 30 min with a fluorography enhancing solution (Amplify, Amersham Biosciences, Inc.), vacuum dried, and subjected to autoradiography.

**RESULTS**

**CBP/p300, SRC-1, and TTF-1 Nuclear Localization Increase in Concert with Alveolar Type II Cell Differentiation and SP-A2 Gene Expression**—CBP/p300 have previously been implicated as having a role in cell differentiation and development (36, 37). To analyze developmental changes in expression, cellular and subcellular localization of CBP/p300, SRC-1, and TTF-1 in human fetal lung in relation to changes in SP-A expression, we utilized midgestation human fetal lung explants maintained in organ culture. As reported previously (43), when midgestation human fetal lung explants are maintained in organ culture in serum-free medium, they differentiate spontaneously and develop the capacity to synthesize surfactant glycerophospholipids (43) and SP-A (12). Differentiation is accelerated when the explants are cultured in the presence of Bt2cAMP (12). As can be seen in Fig. 1, prior to culture (Start), the midgestation human fetal lung was composed of numerous small ducts lined with columnar epithelium and surrounded by abundant mesenchyme (Fig. 1A); immunoreactive SP-A was undetectable (Fig. 1B). Upon organ culture for 1–3 days in serum-free control medium, the ducts progressively enlarged, the epithelium became low cuboidal, and the surrounding mesenchyme was greatly reduced. This was accelerated by Bt2cAMP treatment (Fig. 1A). Immunoreactive SP-A, which was undetectable in the midgestation fetal lung before culture, was first detectable in untreated (control) explants on day 3 of culture and was increased by Bt2cAMP (Fig. 1B).

Parallel sections of the fetal lung tissue were immunostained for TTF-1, CBP, p300, and SRC-1. In contrast to SP-A, immunoreactive TTF-1 was detectable in the ductular epithelium of the human fetal lung tissue prior to culture (Fig. 1A, Start). By immunoblotting, we found that TTF-1 levels increased only modestly during the culture period and, as reported previously (21), were essentially unaffected by Bt2cAMP treatment (data not shown). Interestingly, TTF-1 appeared to be present both in nuclei and cytoplasm of the ductular epithelium before culture.
and after 1 day of incubation in control medium (Fig. 1). By contrast, TTF-1 was predominately localized to the nuclei of epithelial cells of fetal lung explants incubated for 1 day with Bt2cAMP or for 3 days in control or Bt2cAMP-containing medium (Fig. 1).

In the midgestation human fetal lung prior to culture, CBP was present both in the ductular epithelium and in surrounding mesenchyme; within epithelium, CBP appeared to be present both in cytoplasm and nuclei (Fig. 1A). As observed for TTF-1, CBP became more intensely localized to the nuclei of the ductular epithelial cells during differentiation of the fetal lung in culture. Levels of immunoreactive CBP also appeared to increase in the cultured fetal lung as compared with the tissue before culture. Highly similar findings were obtained using antisera specific for p300 (data not shown) and SRC-1; nuclear localization of SRC-1 was enhanced by Bt2cAMP on days 1 and 3 of incubation (Fig. 1A). These findings suggest that CBP/p300, SRC-1, and TTF-1 nuclear localization increase in the fetal lung explants in concert with type II cell differentiation and the induction of SP-A expression.

SP-A2

CBP and SRC-1 Act Synergistically with TTF-1 to Increase SP-A Promoter Activity in a TBE-dependent Manner—The above findings suggest that coactivators CBP/p300 and SRC-1 may play a role together with TTF-1 in the induction of SP-A gene expression that occurs in association with type II cell differentiation. SRC-1 can form a complex with CBP/p300 to synergistically activate transcription of a number of target genes (39, 40). To determine whether CBP and SRC-1 act synergistically with TTF-1 to increase SP-A promoter activity, we utilized a transient transfection assay. A549 lung adenocarcinoma cells were cotransfected with a reporter gene construct containing 255 bp of 5′-flanking sequence from the hSP-A2 gene linked to hGH structural gene, as reporter (bSP-A2-255:hGH). A549 cells are a human lung adenocarcinoma cell line of presumed type II cell origin, which do not express detectable levels of endogenous TTF-1 or SP-A (20, 21). The cells were cotransfected with expression vectors for SRC-1, CBP, and TTF-1 alone and in various combinations; the corresponding empty vectors were cotransfected as controls, where appropriate. Cells also were cotransfected with RSV/β-gal for subsequent analysis of transfection efficiency. Reporter activities were evaluated by radioimmunoassay of hGH in the culture medium, and values were normalized by assay of β-galactosidase activity in the transfected cells. As shown in Fig. 2A, cotransfection of TTF-1 caused a 4- to 5-fold induction of SP-A promoter activity. Neither SRC-1 nor CBP, when transfected individually, had an effect to alter SP-A promoter activity in the absence or presence of cotransfected TTF-1. On the other hand, when CBP and SRC-1 were cotransfected together with TTF-1, they synergistically increased SP-A promoter activity ~14-fold over basal levels. These effects were TBE-dependent, because no stimulation of SP-A promoter activity was evident upon cotransfection of a reporter construct containing a mutation in the most critical TBE element (TBE1) (bSP-A2-255 M1: hGH) (Fig. 2B).

In previous studies, we observed that a (TBE)2:hGH reporter gene construct comprised of a concatamer of three repeats of TBE1 linked to 50 bp of sequence upstream of the transcription start site, and 40 bp of the first exon of the hSP-A2 gene was sufficient to mediate TTF-1 induction of SP-A promoter activity in transfected A549 cells (21). In the present study, A549 cells cotransfected with (TBE)2:hGH, in the absence or presence of an expression vector for the adenoviral protein E1A were analyzed to assess the role of endogenous CBP/p300 in SRC-1 and TTF-1 activation of SP-A promoter activity. Although the precise mechanism remains unknown, E1A is believed to inhibit CBP/p300 function by repressing HAT activity of CBP/p300, as well as the CBP/p300-associated factor (PCAF) (44, 45) and by competing for binding to components of the basal transcriptional machinery, including RNA polymerase II holoenzyme and TFII B (37). Consistent with our previous observations (21), we observed that TTF-1 alone increased (TBE)2:hGH expression ~2- to 3-fold over that of the empty expression vector (Fig. 3). In contrast to our findings with the bSP-A2-255:hGH reporter construct, when the (TBE)2:hGH reporter was used, we consistently found that SRC-1, in the absence of cotransfected CBP, increased bSP-A2 promoter activity ~2-fold as compared with the effect of TTF-1 alone. Cotransfection of the E1A expression vector blocked the capacity of TTF-1 in the absence or presence of SRC-1 to increase SP-A2 promoter activity (Fig. 3). We suggest that this is due to compromising effects of E1A on endogenous CBP/p300 functional activity. These findings suggest that the stimulatory effect of TTF-1 in the absence or presence of cotransfected SRC-1 is dependent upon endogenous CBP/p300. The finding that SRC-1 had no effect to enhance TTF-1 stimulation of bSP-A2-255:hGH expression in the absence of cotransfected CBP may possibly be caused by sequestration of limiting amounts of endogenous CBP/p300 by transcription factors bound to other response elements within the ~255-bp 5′-flanking region. These response elements are absent from the (TBE)2:hGH fusion gene construct. Taken together, these findings suggest that CBP/p300 and SRC-1 play a role together with TTF-1 in transcriptional activation of the SP-A2 promoter.

TTF-1-mediated Stimulation of SP-A Promoter Activity by SRC-1 Is Enhanced by PKA—Several lines of evidence indicate...
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that phosphorylation facilitates interaction of transcription factors with CBP/p300 (37, 46). We previously observed that PKA-mediated phosphorylation of TTF-1 increases its DNA-binding and transcriptional activity (21). To examine the role of PKA on coactivator and TTF-1 activation of SP-A promoter activity, A549 cells were cotransfected with bSP-A2 minimal promoter, linked to hGH, in the absence or presence of expression vectors for TTF-1, SRC-1, and E1A. Shown are the arbitrary units of activity, derived from the corresponding hGH levels secreted into the medium over a 24-h period after transfection. Data are means ± S.E. from two independent experiments, each conducted in triplicate and normalized for transfection efficiency by assay of β-galactosidase activity. The amount and types of plasmid DNAs in all dishes were normalized by transfection with appropriate amounts of the corresponding empty vectors.

of the proteins incubated with the various GST fusion proteins. The two radiolabeled bands in the case of both CBP and SRC-1 may reflect either two alternatively translated products or some type of posttranslational modification in the rabbit reticulocyte lysate system. The radiolabeled SRC-1 or CBP proteins that were retained by the beads were detected by SDS-PAGE and autoradiography. [35S]Methionine-labeled CBP interacted most strongly with the GST fusion protein containing full-length TTF-1, although significant binding to the GST fusion protein containing the N-terminal 207 amino acids of TTF-1 also was observed (Fig. 5A, upper panel). Negligible interactions of radiolabeled CBP were found with GST fusion proteins containing either the homeodomain or the C-terminal 164 amino acids of TTF-1. The modest interaction of CBP with the TTF-1 homeodomain and C-terminal fragment were similar to that observed with GST alone (Fig. 5A, upper panel). When [35S]Methionine-labeled SRC-1 interactions with TTF-1 were evaluated, we observed the strongest binding to the GST fusion protein containing full-length TTF-1. A negligible interaction of radiolabeled SRC-1 with the TTF-1 C-terminal fragment was found, whereas, modest interaction with the TTF-1 N-terminal and homeodomain regions were evident (Fig. 5A, lower panel).

To determine whether endogenously expressed CBP has the capacity to interact with TTF-1, GST pull-down assays were conducted using lysates from A549 cells transfected with RSV:
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CBP or with nuclear extracts from type II cells cultured in Bt2cAMP-containing medium for 3 days. The proteins that interacted with GST and with a GST fusion protein containing full-length TTF-1 were analyzed for CBP by immunoblotting using specific antibodies. As can be seen in Fig. 5B, immunoreactive CBP in lysates of transfected A549 cells and in nuclear extracts of type II cells interacted specifically with GST-TTF-1, but not with GST alone (Fig. 5B).

**SRC-1 Facilitates TTF-1 DNA Binding Activity—**As shown above, TTF-1 interacts with in vitro transcribed/translated SRC-1 and CBP in vitro and appears also to interact with endogenously expressed CBP. Because cAMP/PKA increases TTF-1 DNA-binding activity (21) and also appears to be required for the functional interaction of TTF-1 with SRC-1 and CBP at the TBE (Fig. 4), it was of interest to determine whether interaction of TTF-1 with these coactivators is required for TBE binding. Nuclear extracts from type II cells cultured for 3 days in medium containing Bt2cAMP were incubated with either rabbit or goat nonimmune IgG, as controls, or with antibodies to CBP, SRC-1, or TTF-1. This was followed by incubation with Protein A/G Plus-agarose beads and centrifugation. Equivalent amounts of type II cell nuclear proteins with or without immunodepletion were then incubated with radiolabeled double-stranded TBE probe and analyzed by EMSA, as described above. Alternatively, type II cell nuclear extracts were incubated with the antibodies to CBP, SRC-1, or TTF-1 prior to addition of the radiolabeled TBE probe. As shown in Fig. 6, immunodepletion of SRC-1 or TTF-1 (A), or preincubation with antibody to SRC-1 prior to addition of TBE probe (B), caused a pronounced reduction in TBE-binding activity as compared with binding of type II cell nuclear extracts incubated with nonimmune IgGs or with antibody to CBP. Interestingly, immunoblot analysis of the nuclear extracts immunodepleted of SRC-1 or CBP revealed that the levels of immunoreactive TTF-1 were unchanged as compared with untreated nuclear extracts or those incubated with nonimmune IgG (data not shown). Thus, the decrease in DNA-binding activity by immunodepletion of SRC-1 is not likely due to depletion of TTF-1 protein. On the other hand, it is possible that SRC-1 immunodepletion selectively removed a small component of the total pool of TTF-1 that was activated to a DNA binding state. Nonetheless, our findings suggest that SRC-1 interacts with TTF-1 at the TBE and enhances its DNA-binding activity.

**Cyclic AMP Stimulates TTF-1 Acetylation—**CBP/p300 have the capacity to increase the acetylation of p53 and HNF-4, resulting in an increase in their DNA-binding activities (48, 49). Because we previously observed that Bt2cAMP treatment of type II cells increased TBE-binding activity (21), it was of interest to analyze the effect of cAMP on TTF-1 acetylation. Type II cells that had been cultured in the absence or presence of Bt2cAMP for 3 days were metabolically labeled with [3H]acetate for 2 h; TTF-1 was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. As can be seen in Fig. 7 (top panel), the incorporation of [3H]acetate into TTF-1 was markedly induced by Bt2cAMP treatment. The increase in TTF-1 acetylation in the type II cells was associated with cAMP induction of SP-A protein levels (bottom panel), although the levels of immunoreactive TTF-1 were unaffected (middle panel).

**DISCUSSION**

The homeodomain transcription factor, TTF-1/Nkx2.1, plays a critical role in lung branching morphogenesis (28) and in cAMP regulation of SP-A gene expression (21). Whereas, TTF-1 is expressed from the very earliest stages of lung development (26), SP-A transcription is initiated only after ~70% of gestation is completed (6). Therefore, it is likely that other factors also play an important role in developmental, cell-specific, and...
hormonal regulation of SP-A gene expression. These may include developmental changes in TTF-1 posttranslational modification, nuclear localization, interaction with other transcription factors critical for regulation of SP-A expression, and/or recruitment of coactivators. Previously, we observed that cAMP/PKA stimulation of SP-A expression is associated with increased TTF-1 phosphorylation, DNA binding to the TBE, and transcriptional activity (21). To begin to define the molecular mechanisms that modulate TTF-1 actions on lung morphogenesis and function, we have analyzed the potential role of coactivators and their interactions with TTF-1 in developmental and cAMP regulation of SP-A gene expression.

In the present study, we observed that nuclear localization of TTF-1 increased in the human fetal lung explants in association with type II cell differentiation. Furthermore, after the first 24 h of culture, TTF-1 nuclear localization was enhanced by cAMP treatment, as compared with explants maintained in control medium. On the other hand, total cellular levels of immunoreactive TTF-1, analyzed by immunoblotting, only modestly increased in the fetal lung tissue during differentiation in culture in the absence or presence of Bt2cAMP (data not shown). In previous studies using differentiated human type II cells in primary culture, we found that cAMP treatment increased TTF-1 phosphorylation and DNA-binding and transcriptional activity (21). Essentially all of the immunoreactive TTF-1 was recovered in nuclei-enriched fractions, and TTF-1 levels were unaffected by cAMP treatment. This suggests that, once type II cell phenotypic differentiation is achieved, TTF-1 is predominately localized to the nucleus. The finding in H441 lung adenocarcinoma cells that treatment with transforming growth factor-β (50) or phorbol ester (51) caused cytoplasmic trapping of TTF-1, suggests that factors that cause cellular transformation/de-differentiation may prevent TTF-1 accumulation in the nucleus. Thus, shutting of TTF-1 between the cytoplasm and nucleus may play an important role in regulation of its transcriptional activity.

In the present study, we also made the novel observation that nuclear localization of the coactivators, CBP, p300, and SRC-1, increased in pre-alveolar duct epithelial cells of the midgestation human fetal lung explants in concert with type II cell differentiation. These findings suggest that these coactivators may play a key role together with TTF-1 in the onset of SP-A gene expression and are consistent with the suggested importance of CBP/p300 in embryonic differentiation and development (36, 37).

In transient transfection assays of A549 lung adenocarcinoma cells using a reporter gene construct containing 255 bp of 5′-flanking DNA from the bSP-A2 gene linked to hGH, we found that CBP and SRC-1, in combination, acted synergistically with TTF-1 in a TBE-dependent manner to increase bSP-A2 promoter activity. When this native promoter construct was utilized, neither CBP nor SRC-1, when coexpressed alone with TTF-1, enhanced bSP-A2 255-hGH expression. However, coexpression of PKAcat with SRC-1 and TTF-1 mimicked the synergistic stimulation of bSP-A2 promoter activity observed upon coexpression of CBP. This suggests that PKAcat-mediated phosphorylation of TTF-1 may facilitate the recruitment of endogenous CBP/p300 to the complex with SRC-1 resulting in promoter activation. This finding is of interest, because CBP and p300 were initially recognized as coactivators involved in regulation of cAMP-inducible promoters. PKA-mediated phosphorylation of the transcription factor CREB facilitates the recruitment of CBP/p300, which in turn, interacts with the basal transcription complex (35, 36, 52). cAMP also has been reported to increase phosphorylation of SRC-1 on two mitogen-activated protein kinase sites, which in turn enhances the functional cooperation of SRC-1 with CBP and activation of gene transcription (53). The importance of endogenous CBP/p300 in TTF-1 and SRC-1 induction of SP-A promoter activity was emphasized by our finding that coexpression of the adenoviral protein E1A blocked both PKAcat and SRC-1 stimulation of bSP-A2 promoter activity when A549 cells were cotransfected with TTF-1 and the (TBE)7-hGH reporter gene construct. E1A apparently blocks CBP/p300 function by inhibiting HAT activity (44, 45) or by disrupting its interaction with SRC-1 (54).

In GST pull-down assays, we observed that TTF-1 has the capacity to interact with SRC-1 and CBP in vitro. Furthermore, endogenous CBP in nuclear extracts of cAMP-treated human fetal type II cells or in A549 cells transfected with a CBP expression vector, also interacted specifically with GST-TTF-1. As mentioned above, CBP/p300 and SRC-1 activate transcription, in part, through intrinsic HAT activity resulting in acetylation of histone tails, the local unwinding of nucleosomes and loosening of the higher order chromatin structure surrounding the promoter. This facilitates recruitment to the promoter of general transcription factors and RNA polymerase II, resulting in stabilization of the preinitiation complex and activation of transcription initiation (33, 55–57). Recent studies indicate that CBP/p300 may also increase transcription by direct acetylation of certain transcription factors (48, 49, 58). Increased acetylation of p53 and HNF-4 transcription factors by CBP/p300 increased their DNA-binding activity, nuclear retention, stability, and affinity of interaction with CBP (48, 49, 59).

Because we previously observed that Bt2cAMP treatment of type II cells increased TTF-1 phosphorylation and DNA-binding activity (21), it was of interest to analyze the effect of cAMP on TTF-1 acetylation. In the present study, we made the intriguing observation that cAMP treatment of type II cells
caused a marked induction in the rate of TTF-1 acetylation. The cAMP-induced increase in TTF-1 acetylation was associated with an increase in SP-A expression, whereas the levels of immunoreactive TTF-1 were unaffected. Although acetylation of transcription factors by SRC-1 has never been reported, TTF-1 could possibly serve as a substrate for SRC-1 HAT activity. Alternatively, SRC-1 may coordinate TTF-1 acetylation by CBP/p300 through its stable interaction with both proteins. In future studies it will be important to identify the one or more cAMP-induced acetylation sites in TTF-1, determine whether acetylation is required for increased TBE-binding activity, and determine whether TTF-1 serves as a substrate for SRC-1 HAT activity. Interestingly, a KRR sequence found to be a critical acetylation motif in the transcription factor GATA-3 has been identified in the homeodomain of TTF-1 (60). In a topology study of the TTF-1 homeodomain, it was observed that selective acetylation of lysine residues changed the surface accessibility upon forming a complex with DNA (61). This suggests that TTF-1 acetylation may significantly change its interface with DNA and/or with other proteins.

Based on past and present findings, our current view of the mechanisms for cAMP induction of SP-A gene expression is presented in Fig. 8. We previously observed that cAMP induction of SP-A promoter activity in lung type II cells requires the cooperative interaction of transcription factors bound to a number of critical response elements. These factors include a putative, but as yet unidentified, member of the nuclear receptor family, USFs 1 and 2, Sp1 and related members of the Krüppel family, and TTF-1 (see Ref. 16 for review). During lung development and type II cell differentiation, endogenous and exogenous signals activate PKA, which subsequently increases levels of cAMP. PKA, resulting in the release and activation of the catalytic (C) subunits. cAMP/PKA advances the program of type II cell differentiation, which may involve increased nuclear localization of TTF-1 and the coactivators SRC-1 and CBP/p300. PKA catalytic subunits catalyze the phosphorylation of TTF-1, which enhances its ability to bind to the TBE. Phosphorylated TTF-1 bound to the TBE recruits a complex of coactivators, including SRC-1 and CBP, which contain intrinsic HAT activity. The HAT associated with these coactivators catalyzes the acetylation of histones within chromatin, resulting in a local unwinding of chromatin structure, which facilitates the binding of other transcription factors to critical response elements within the SP-A gene 5′-flanking region and of components of the basal transcription complex to the TATA-box. The HAT also catalyzes the acetylation of lysine residues in TTF-1, which may further increase its nuclear retention, DNA binding, and cooperative interaction with other transcription factors, leading to increased SP-A gene transcription. The stimulatory effects of cAMP on type II cell differentiation and SP-A gene expression are dependent upon a critical environmental O2 tension (63). We postulate that enhanced vascularization of the fetal lung during the latter third of gestation with consequent increased O2 tension within the pre-alveolar epithelium serves a permissive role in the cascade of molecular events leading to type II cell differentiation and activation of SP-A gene expression.

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