Temporal/spatial expression of nuclear receptor coactivators in the mouse lung

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Naltner, Angela, Susan Wert, Jeffrey A. Whitsett, and Cong Yan. Temporal/spatial expression of nuclear receptor coactivators in the mouse lung. Am J Physiol Lung Cell Mol Physiol 279: L1066–L1074, 2000.—Our laboratory has previously demonstrated that retinoic acid nuclear receptor, thyroid transcription factor-1 (TTF-1), and nuclear receptor coactivators such as cAMP response element binding protein (CREB) binding protein (CBP)/p300 and steroid receptor coactivator-1 (SRC-1) form an enhanceosome on the 5′-enhancer region of the human surfactant protein B gene. Immunohistochemistry was used to identify cells that coexpressed CBP/p300, SRC-1, retinoid X receptor, and TTF-1 in the developing and mature lung. CBP/p300 and SRC-1 were expressed in the adult mouse lung, CBP and p300 being present in both alveolar type I and type II epithelial cells and SRC-1 and TTF-1 being restricted to type II epithelial cells. CBP/p300, SRC-1, and TTF-1 were readily detected in the nuclei of developing respiratory epithelial tubules in fetal mice from embryonic days 10 to 18. CBP/p300 and SRC-1 were also detected in developing mesenchymal cells. These coactivators were coexpressed with TTF-1 and SP-B in human pulmonary adenocarcinoma cells (H441 cells) in vitro. Interaction assays with a two-hybrid reporter analysis demonstrated direct interactions among TTF-1, SRC-1, and CBP/p300 in H441 cells. These findings support a role for retinoic acid receptor and nuclear receptor coactivators in the regulation of SP-B gene expression in the respiratory epithelium.

thyroid transcription factor-1; steroid receptor coactivator-1; cyclic adenosine 5′-monophosphate response element binding protein binding protein/p300; surfactant protein B; lung development

SIGNALLING VIA THE RETINOIC ACID (RA)/retinoic acid receptor (RAR) axis is known to mediate epithelial cell differentiation and proliferation in numerous tissues including the lung (9, 13). The absolute requirement of RAR signaling to lung morphogenesis was observed in double-knockout RAR-α-deficient and RAR-β-deficient mice that developed lung hypoplasia and aplasia (16). Previous studies (1, 14, 15, 17) indicated that RA influenced branching morphogenesis and alveolarization of the fetal lung in vitro. RA enhances surfactant protein B (SP-B) mRNA and SP-B expression in lung epithelial cells and explant cultures of fetal lungs (1, 4, 5, 17, 30). SP-B is a 79-amino acid peptide required for respiratory adaptation after birth. SP-B enhances the spreading and stability of phospholipids in surfactant in the alveoli (28). SP-B is expressed in alveolar type II epithelial cells and in subsets of nonciliated bronchiolar cells that line conducting airways; it is also found in H441 (human pulmonary adenocarcinoma) cells in vitro. Transcription of the SP-B gene is dependent on cis-acting elements in the 5′-flanking regulatory region of the gene. Studies of the human (h) SP-B 5′-flanking regulatory region in H441 cells identified an enhancer located at −500 to −375 bp (31). Deletion of the enhancer region significantly impaired the transcription activity of the hSP-B gene. A subsequent study (18) identified clustered RA-responsive element (RARE) sites located in the enhancer region (18). The RARE sites mediate stimulation of the RA effect on expression of the hSP-B gene; deletion or mutation of the RARE sites reduced or abolished stimulation by RA. RAR and its coactivators (activator of thyroid and RA receptor (ACTR), steroid receptor coactivator (SRC)-1, transcriptional intermediary factor-2, and cAMP response element binding protein (CREB) binding protein (CBP)) stimulated activity of the hSP-B promoter in a dose-dependent fashion (18). The finding that expression of a dominant negative RAR mutant protein inhibited hSP-B transcription demonstrated that RAR signaling was required for full activity of the SP-B promoter in pulmonary epithelial cells in vitro (6).

Although RARs are known to influence epithelial differentiation and proliferation in various organs including the lung, the interactions between RAR and tissue-specific factors in the lung are poorly understood. In a previous study (18), RA stimulation of the hSP-B gene was not only dependent on RARE but was also dependent on the binding of thyroid transcription factor-1 (TTF-1) to the adjacent sites in the enhancer of the hSP-B gene. TTF-1 is a tissue-specific transcription factor of the Nkx2 family expressed in the epithelial cells of the lung, thyroid, and part of the forebrain (11). Lung morphogenesis and surfactant protein expression were markedly disrupted in TTF-1-deficient mice (9a). Mammalian two-hybrid and cotransfection studies demonstrated that TTF-1 interacted with RAR, synergistically stimulating transcription of SP-B by...
interacting with RAR in H441 cells (18). Stimulation of target gene expression by RAR is mediated by recruitment of p160 coactivator members (SRC-1, transcriptional intermediary factor-2, and ACTR), CBP/p300, and other coactivators to form a transcription complex (3, 7, 8, 12, 20, 25, 27). In pulmonary epithelial cells, RAR coactivators activated hSP-B transcription activity in a synergistic manner with TTF-1 (18). These studies support a model wherein RAR/retinoid X receptor (RXR), TTF-1, and nuclear receptor coactivators form an enhanceosome in the enhancer region of the hSP-B gene (Fig. 1).

This model depends on the coexpression of RAR/RXR, coactivators, and TTF-1 with SP-B in respiratory epithelial cells. In the present study, immunohistochemistry was used to demonstrate temporal/spatial colocalization of the coactivators SRC-1, CBP/p300, and TTF-1 in developing and adult lungs and in H441 pulmonary adenocarcinoma cells. Direct protein-protein interactions among TTF-1, SRC-1, and CBP were demonstrated in a mammalian two-hybrid reporter system in vitro.

**Materials and Methods**

**Plasmids.** The plasmid constructs of pVP16AD/TTF-1, pVP16AD/SRC-1, and pMBD/SRC-1 were made by subcloning the PCR products of TTF-1 and SRC-1 molecules into the pVP16 activation domain (AD) and pM binding domain (BD) vectors (Clontech, CA) at the EcoRI/XbaI sites for TTF-1 and the MluI/XbaI sites for SRC-1. The PCR products and empty vectors were purified by low-melting-point agarose gel electrophoresis and the Qiagen gel extraction kit and then digested by their respective enzymes. Ligations of digested PCR products and vectors were transformed into DH5α cells, and the correctness of the constructs was confirmed by DNA sequencing. Plasmid pM-53 BD and pVP16-CP AD were purchased from Clontech. CBP-BD was a kind gift from Dr. R. Evans (Salk Institute, San Diego, CA).

**Cell culture.** H441 cells were cultured in RPMI 1640 medium supplemented with 10% FCS, glutamine, and penicillin-streptomycin. Cells were maintained and passaged weekly at 37°C in 5% CO₂-air.

**Mammalian two-hybrid system assay.** Transient transfections of the mammalian two-hybrid constructs were performed as described previously (18). H441 cells were seeded at a density of 3 × 10⁴ cells/well in a six-well plate and incubated at 37°C in 5% CO₂-95% air. The paired AD-BD constructs were transfected along with pCMV/β-gal and PG5Luciferase reporters (18), all at 0.5 μg with Fugene 6 (Roche/Boehringer Mannheim). The cells were harvested 48 h after transfection and assayed by luminometry (Monolight 3010, Analytical Luminescence Laboratory). β-Galactosidase activities were also determined for each transfection for normalization purposes.

**Immunocytochemistry.** H441 cells were seeded on two-well Permanox chamber slides (Nalge Nunc) at densities of 2.5 × 10⁴ to 5.0 × 10⁵ cells/chamber. Cells were washed twice in PBS and fixed with 4% paraformaldehyde for 10 min. Cells were washed in PBS, permeabilized in methanol, and rinsed in PBS. Antigen retrieval was performed on the cells by microwave at a 100% energy level for 7.5 min, followed by three exposures of 5 min each at a 60% energy level. Cells were washed in 0.1 M PBS with 3% Triton X-100. After the nonspecific binding was blocked, primary antibodies were used.
added to the cells. The dilutions of the antibodies used were 1:500 for SRC-1 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA); 1:1,000 for TTF-1 rabbit polyclonal antibody (Dr. R. Di Lauro, Stazione Biologic, Naples, Italy); and 1:500 for CBP rabbit polyclonal antibody and p300 rabbit polyclonal antibody (Santa Cruz Biotechnology). The cells were incubated at 4°C with primary antibody overnight, washed the following day, and treated with secondary conjugated antibody. Cells were washed and stained with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Staining was developed by a series of incubations in nickel-3,3′-diaminobenzidine (DAB) and Tris-cobalt and counter-

Fig. 3. Immunohistochemical staining of TTF-1, RXR, SRC-1, CBP, and p300 in the adult lung. Sections from adult mouse lung were stained with TTF-1 antibody (1:10,000), RXR antibody (1:2,000), SRC-1 antibody (1:100), CBP antibody (1:500), and p300 antibody (1:500) as described in MATERIALS AND METHODS. Br, bronchioli. Original magnification, ×920.

Fig. 4. Immunohistochemical staining of TTF-1 in fetal lungs. Sections of fetal mouse lungs from embryonic day 10 (E10), E12, E14, E16, and E18 were stained with TTF-1 antibody (1:5,000) as described in MATERIALS AND METHODS. Di, diverticulum; ET, epithelial tubules. Original magnification: E10 and E12, ×287; E14, E16, and E18, ×575.
stained with nuclear fast red, and coverslips were applied. In the control experiment, nonimmune serum revealed no staining of H441 cells.

Immunohistochemistry. Tissue sections were prepared from wild-type adult mouse lungs and from fetal mouse lungs on embryonic days (ED) 10, 12, 14, 16, and 18. Briefly, wild-type mice were mated, and the pregnant mice were killed on ED10, ED12, ED14, ED16, and ED18. The pups were immersed in a fixative of 4% paraformaldehyde and PBS. The pups were left in the fixative at 4°C overnight and then were processed through a series of ethanol washes overnight. The pups were then embedded in paraffin and sections were prepared. Slides were baked at 60°C for a minimum of 2 h and washed in a series of xylene and ethanol to remove paraffin from the tissues. Antigen retrieval was performed on the tissues as described in Immunocytochemistry. Endogenous peroxidase activity was removed from the tissues by incubating them in methanol and hydrogen peroxide for 15 min. After a wash in 0.1 M PBS with Triton X-100, nonspecific binding was blocked by incubating the slides in 0.1 M PBS with Triton X-100 with either goat or rabbit serum for 2 h, depending on the antibody used. The slides were then incubated overnight at 4°C in primary antibody. The dilutions of primary antibodies used were 1:2,000 for RXR-α rabbit polyclonal antibody (Santa Cruz Biotechnology) for adult lung; 1:100 for SRC-1 for adult lung and 1:50 for ED12–ED18; 1:10,000 for TTF-1 for adult lung, 1:5,000 for ED10, 1:15,000 for ED12, and 1:1,000 for ED14–ED18; 1:500 for CBP for adult lung, 1:200 for ED12–ED18; and 1:500 for p300 for adult lung and 1:100 for ED12–ED18. The tissues were washed and treated with secondary conjugated antibodies 24 h later. After the washes, the interaction was amplified with Vectastain Elite ABC kit. After being washed, the tissue staining was developed by a series of incubations in nickel-DAB and Tris-cobalt and counterstained with nuclear fast red. The tissues were finally run through a series of dehydration steps in alcohols and xylenes before coverslips were added. In control experiments, nonimmune serum revealed no staining of tissue sections.

RESULTS

Expression of SRC-1, CBP/p300, and TTF-1 in H441 cells. RAR/RXR, TTF-1, and coactivators synergistically stimulated the activity of the hSP-B gene promoter in H441 cell transfection assays (18). It is important, therefore, to demonstrate that these factors are colocalized in H441 cells. Previously, RAR and RXR were detected by immunohistochemistry in H441 cells (30). In the present study, antibodies against TTF-1, SRC-1, CBP, and p300 were used to immunostain H441 cells. Nuclear staining for TTF-1, SRC-1, CBP, and CBP homolog p300 was detected in the nuclei of H441 cells (Fig. 2).

Coexpression of SRC-1, RXR, CBP/p300, and TTF-1 in adult mouse lung. Expression of the SP-B gene is restricted to distal bronchiolar and type II epithelial cells in the lung. In addition to the H441 cell line, colocalization study of TTF-1, RAR/RXR, and coactivators in bronchiolar and type II epithelial cells is critical.
to the understanding of the mechanism by which expression of the SP-B gene is regulated in the physiological condition in vivo. As previously reported (18, 30), RAR was expressed in bronchiolar and alveolar epithelial cells in adult mouse lung. Sections from adult mouse lungs were prepared and immunostained with antibodies against TTF-1, RXR, SRC-1, CBP, and p300. Interestingly, staining for TTF-1, RXR, and SRC-1 in the peripheral region of the mouse lung was restricted to bronchiolar and alveolar type II epithelial cells (Fig. 3). CBP and p300, on the other hand, were detected in all cells in the peripheral region of the lung (Fig. 3), including stromal and epithelial cells. Therefore, RAR, RXR, TTF-1, SRC-1, and CBP/p300 are coexpressed in bronchiolar and alveolar type II cells, which are known to be sites of SP-B synthesis.

**TTF-1 expression in mouse lung development.** Expression of the SP-B gene is developmentally regulated in the lung. Temporal/spatial expression of TTF-1, RAR/RXR, and coactivators that are critical to SP-B promoter stimulation need to be assessed in the developing lung. The temporal/spatial distribution of TTF-1 was assessed first in lungs from fetal mice. Tissue sections were prepared from lungs of embryos on ED10, ED12, ED14, ED16, and ED18 and were stained with TTF-1 antibodies. TTF-1 expression was detected as early as ED10 in the developing mouse lung and was restricted to cells lining epithelial tubules throughout lung development (Fig. 4), consistent with results of a previous study (11). At ED18, TTF-1 was detected in alveolar type II epithelial cells in the lung, in agreement with previous observations (32) that TTF-1 and SP-B were colocalized during fetal lung development.

**SRC-1 expression in developing mouse lung.** The temporal/spatial expression of SRC-1 was studied during lung morphogenesis. Lung tissue sections from fetal mice on ED12, ED14, ED16, and ED18 were stained with SRC-1 antibody. SRC-1 was detected in epithelial tubules throughout lung development (Fig. 5). In addition, SRC-1 was detected in certain pulmonary mesenchymal cells (Fig. 5), suggesting potential roles for SRC-1 in both the epithelium and the mesenchyme.

**CBP and p300 expression in mouse lung development.** CBP and p300 are homologs and are required for multiple intracellular signaling pathways, including the RA/RAR axis pathway. Mouse tissue sections from ED12, ED14, ED16, and ED18 were stained with the CBP and p300 antibodies (Figs. 6 and 7). Nuclear staining for both CBP and p300 was present in almost all types of cells at various stages of lung development. However, the intensities of immunostaining varied among different cells, an observation consistent with the roles of CBP and p300 as general coactivators participating in multiple signaling pathways and mediating cell proliferation and differentiation.

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Fig. 6. Immunohistochemical staining of CBP in fetal lungs. Sections from E12, E14, E16, and E18 fetal mouse lungs were stained with CBP antibody (1:200) as described in MATERIALS AND METHODS. Original magnification: E12, ×287; E14, E16, and E18, ×575.
Interactions among TTF-1, SRC-1, and CBP in H441 cells. Not only do TTF-1, RAR/RXR, and coactivators stimulate SP-B gene expression, they are also colocalized in the developing epithelial cells throughout lung development and in bronchiolar and type II epithelial cells in the mature lung. It is therefore intriguing to establish interactions among these key players for stimulation of the SP-B promoter. Previously (18), interactions between TTF-1 and RAR were observed with the mammalian two-hybrid system in H441 cells. To assess whether the coactivator SRC-1 and CBP interact with TTF-1, “bait” was prepared, including SRC-1 AD and TTF-1 AD, in which TTF-1 and SRC-1 were fused with the VP16 AD in the pVP16 vector. The “prey” were SRC-1 BD and CBP BD, in which SRC-1 and CBP were fused with the GAL4 BD. The luciferase reporter construct pG5LUC containing five GAL4 binding sites and an adenovirus E1b minimal promoter was cotransfected with construct pairs of TTF-1 AD/SRC-1 BD, TTF-1 AD/CBP BD and SRC-1 AD/CBP BD into H441 cells. To serve as controls, the construct pG5LUC was also cotransfected with each individual plasmid. Increased luciferase activity was observed (data not shown). It is known that p53 does not interact with a polyoma virus coat protein. Therefore, TTF-1 interacts not only with RAR but also with SRC-1 and CBP in H441 cells. The interactions among these factors strongly suggest that TTF-1, RAR/RXR, and coactivators form an enhanceosome in the SP-B enhancer region (~500 to ~375 bp) that is required for SP-B gene activation in lung epithelial cells.

DISCUSSION

The steroid/nonsteroid nuclear receptor superfamily is composed of a large number of proteins that bind lipophilic hormones as ligands. The binding of ligands triggers conformational changes of the nuclear receptors that subsequently bind to hormone-responsive elements on the target genes as heterodimers or homodimers. The nonsteroid hormone nuclear receptors such as RAR and thyroid receptor form heterodimers with RXR, whereas the glucocorticoid receptor forms homodimers. On binding to hormone-responsive elements of the target genes, nuclear receptors start to recruit a number of coactivators, including p160 members (SRC-1/NeuA-1, transcriptional intermediary factor-2/GRIP1/NeuA2, ACTR/pCIP/AIB1) and CBP/p300, to form transcription activation complexes (3, 7, 8, 12, 20, 25, 27). Many of these coactivators activate gene transcription through two steps (10). First, CBP,
SRC-1, and ACTR have intrinsic histone acetyltransferase (HAT) activities. Once bound to the hetero- or homodimers of nuclear receptors, these coactivators acetylate histones. Nucleosomes undergo conformational changes that lead to chromosome loosening, which, in turn, opens up the promoter region and allows assembly of the basic transcriptional machinery. Second, on binding to the enhancer and the promoter regions of a gene, nuclear receptors, coactivators, and basic transcription factors directly interact to cause conformational changes of the preinitiation transcription complex to a more stable form, facilitating gene transcription and elongation.

Functional studies in vitro established a model in which the stimulatory effects of RA on SP-B gene expression are influenced by an enhanceosome containing RAR, TTF-1, and coactivators. Nucleosomes undergo conformational changes that lead to chromosome loosening, which, in turn, opens up the promoter region and allows assembly of the basic transcriptional machinery. Second, on binding to the enhancer and the promoter regions of a gene, nuclear receptors, coactivators, and basic transcription factors directly interact to cause conformational changes of the preinitiation transcription complex to a more stable form, facilitating gene transcription and elongation.

The reversible acetylation of specific lysine residues within the N-terminal tails of core histones is associated with gene transcription. A recent study showed that HAT proteins are part of large multiprotein complexes. For example, CBP/p300-associated factor (P/CAF) is associated with TATA binding factor.
associated factor (TAF) and TAF-related subunits and transcription-associated protein (TRRAP) (19, 26). The yeast counterpart SAGA (Spt-Sda-Gen5-acetyltransferase) complex is composed of at least 14 subunits (2). Other HAT complexes include TFIIC (basal transcription factor III C complex), TFIID (basal transcription factor II D complex), NuA3 (nucleosomal acetyltransferase of histone H3), NuA4 (nucleosomal acetyltransferase of histone H4), and Elongator (2). The number of HATs and distinct HAT complexes that have been identified is large and still growing. How these HATs selectively affect gene expression is an intriguing question. It has been shown that pituitary-specific factor-1-dependent transcription requires CBP HAT activity when activated by cAMP, whereas it requires P/CAF HAT activity when activated by growth factors (29). Taken together with the present and previous findings (18, 32), CBP and SRC-1 HAT activities not only depend on RAR/RXR binding but also interact with tissue-specific factor TTF-1 to form a complex and activate hSP-B gene transcription in pulmonary epithelial cells. TTF-1, RAR/RXR, SRC-1, and CBP/p300 are colocalized with SP-B expression in both adult pulmonary epithelial type II cells and developing pulmonary epithelial tubules. Therefore, the model of a transcriptional complex demonstrated in H441 cells may play a role in the developing lung and in the adult lung in vivo. The finding that the function of RAR and coactivators depends on the tissue-specific transcription factor TTF-1 shows a novel mechanism by which epithelial cells selectively control gene expression in the pulmonary system.

Differential temporal/spatial expression of TTF-1, RAR/RXR, SRC-1, and CBP/p300 indicates that they may play broader roles in gene expression, alveolarization, and morphogenesis in the lung. It has been shown before (21–23) that supplementation of vitamin A reduced bronchopulmonary dysplasia-associated morbidity in preterm infants. Elucidation of expression patterns and interactions among these factors provide significant insight into the mechanism by which RA, a vitamin A derivative, regulates SP-B gene expression, reduces bronchopulmonary dysplasia, and facilitates respiratory epithelial recovery after oxygen injury.

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