Protein-Protein Interaction of Retinoic Acid Receptor α and Thyroid Transcription Factor-1 in Respiratory Epithelial Cells*

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Surfactant protein B (SP-B) is a 79-amino acid peptide critical to postnatal respiratory adaptation and is developmentally regulated. Previous studies demonstrated that retinoic acid receptors (RARs) and thyroid transcription factor 1 (TTF-1) stimulated SP-B gene expression in respiratory epithelial cells. Clustered retinoic acid-responsive element and TTF-1 binding sites were identified in the enhancer region of the SP-B gene and were required for retinoic acid stimulation of the human SP-B (hSP-B) promoter. In addition, RAR and TTF-1 were colocalized in mouse bronchiolar and alveolar type II epithelial cells, the cellular site of SP-B synthesis. In the present studies, RAR and TTF-1 were colocalized in the nucleus of H441 cells. RAR and TTF-1 synergistically stimulated the hSP-B promoter in H441 cells. Direct protein-protein interactions between RAR and TTF-1 were demonstrated by the glutathione S-transferase pull-down assay and the mammalian cell two hybrid assay. Truncation/deletion studies showed that the RAR-TTF-1 interaction was mediated through the RAR DNA binding domain (DBD) and the TTF-1 homeodomain. RAR DBD greatly enhanced TTF-1 homeodomain DNA binding activity to a hSP-B enhancer oligonucleotide, in which retinoic acid-responsive element and TTF-1 DNA binding sites overlap. Chromatin immunoprecipitation assay demonstrated that retinoic acid treatment of H441 cells greatly stimulated both RAR and TTF-1 DNA binding to the hSP-B enhancer region in H441 cells. These findings support a model in which RAR/retinoid X receptor, TTF-1, and coactivators (p160 members and CBP) form an enhancing complex in the enhancer region of the hSP-B gene.

Signaling via the retinoic acid (RA)/retinoic acid receptor (RAR) axis is known to be important to epithelial cell differentiation and proliferation in the lung (1). RAR signaling is required in lung morphogenesis as observed in double knockout RARα−/− and RARβ−/− mice that developed lung hypoplasia and aplasia (2). RA influenced branching morphogenesis and alveolarization of the fetal lung in vitro (3–5). RA enhanced SP-B mRNA and surfactant protein B (SP-B) expression in lung epithelial cells and explant cultures of fetal lungs (3, 5–8). SP-B is produced in alveolar type II epithelial cells and in subsets of non-ciliated bronchiolar cells lining conducting airways, and in H441 cells (human pulmonary adenocarcinoma cells). SP-B enhances the spreading and stability of phospholipids in surfactant in the alveoli and plays a critical role in lamellar body and tubular myelin organization (9). SP-B is essential for postnatal respiratory adaptation after birth. Mutations of the SP-B gene in the human and mouse cause lung dysfunction at birth and susceptibility to oxygen toxicity (10–13).

An enhancer located at –500–375 base pairs was identified in the hSP-B 5′-flanking regulatory region that contains clustered retinoic acid-responsive elements (RAREs) and TTF-1 binding sites (14, 15). Deletion of the enhancer sequence significantly reduced transcriptional activity of the hSP-B promoter (14). These sites were required for RA stimulation of hSP-B gene expression in respiratory epithelial cells. Both RAR and TTF-1 bound to the clustered RARE and TTF-1 binding sites in the enhancer region of the hSP-B gene (14, 15). A dominant negative RAR mutant inhibited hSP-B transcription (16).

RAR belongs to the steroid/nonsteroid nuclear hormone receptor superfamily and consists of three receptor isotypes α, β, and γ, which are encoded by distinct genes. RAR forms a heterodimer with retinoid X receptor (RXR) that binds to RARE on the target genes. Whereas RAR has weak DNA binding affinity, RXR greatly enhances RAR DNA binding affinity through dimerization of RAR/RXR (17). RAR consists of a DNA binding domain containing Zn2+ finger motifs, a ligand-binding/dimerization domain, a ligand-independent AF-1 transcription activation domain, and a ligand-dependent AF-2 transcription activation domain. Through these various functional domains, RAR interacts with other transcription factors and coactivators to stimulate gene transcription.

TTF-1 is a tissue-specific transcription factor of Nkx2 family members expressed in the lung, the thyroid, and part of the forebrain (18). In the lung, TTF-1 mRNA and protein were detected at the earliest stages of differentiation and were restricted to bronchial and alveolar epithelium in the postnatal lung (18, 19). TTF-1 binds to and activates the promoters of a number of genes selectively expressed in the respiratory epithelium, including SP-B (20). Lung morphogenesis and surfactant protein expression were markedly disrupted in TTF-1−/− mice (21). Studies by deletion/truncation mutagenesis, mammalian cell cotransfection, electrophoretic mobility shift assay, and immunofluorescent assays revealed three distinct functional domains of TTF-1 (22). The N- and C-terminal regions of TTF-1 are transactivation domains. The homeodomain (HD) of TTF-1 is responsible for DNA binding and nuclear localization. RA treatment triggers formation of an enhancing complex in the
hSP-B enhancer region that contains RAR/RXR, TTF-1, CBP, and p160 coactivators (19). The DNA binding of RAR and TTF-1 to the hSP-B enhancer plays a critical role for enhanceosome formation. Because both clustered RAR and TTF-1 DNA binding sites overlap in the enhancer region of the hSP-B gene, it is highly possible that RAR and TTF-1 interact with each other to facilitate enhanceosome formation. In this report, direct interactions between TTF-1 and RAR were identified that enhanced DNA binding to the hSP-B enhancer in respiratory epithelial cells.

**MATERIALS AND METHODS**

**Cell Culture**—Human pulmonary adenocarcinoma cells (H441) were cultured in RPMI supplemented with 10% fetal calf serum, glutamine, and penicillin/streptomycin. Cells were maintained at 37 °C in 5% CO2/air and passed weekly.

**Co-localization of RAR and TTF-1 in H441 Cells by Immunofluorescent Double Staining Assay**—The hRARαs (from Dr. Pierre Chambon) and TTF-1-FLAG (22) expression vectors were cotransfected into H441 cells. Immunofluorescent staining was performed 2 days after cotransfection following a procedure described previously (22). TTF-1-FLAG was recognized by FLAG monoclonal antibody (Eastman Kodak Co.) conjugated with fluorescein isothiocyanate, whereas hRARαs were recognized by anti-hRARα polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) conjugated with Texas red. The fluorescent signals were analyzed by the Leica DM IRBE confocal microscope.

**Transfection and Reporter Gene Assays**—The hSP-B-500 construct was made previously (14). The RAR expression vector was from the original authors (Dr. Pierre Chambon). For the RAR/TTF-1 cotransfection study, transient transfection and luciferase reporter assays were performed as described previously (14, 15). Briefly, H441 cells were seeded at a density of 1 × 10^5 cells/well in six-well plates. The hSP-B500 reporter construct (0.25 µg) was cotransfected with 0.5 µg of RAR, 0.5 µg of PCRS3/0-TTF-1, and 0.5 µg of pCMV-β-galactosidase plasmid into H441 cells for 48 h. The protein gels were fixed and incubated with the luciferase assay system (Promega). The light units were determined for normalization of transfection efficiency.

**Glutathione S-Transferase (GST) Pull-down Assay**—The full-length and various truncated TFF-1 constructs were from a previous study (22). The C-terminal domain was subcloned into the pGEX-4 vector (Invitrogen, San Diego, CA) at the Xho I (from Dr. Pierre Chambon) and TTF-1 BD constructs were made previously (15). The plasmids of constructs were cotransfected into H441 cells. The C-terminal domain was subcloned into the PCR3.0 vector and various truncated TTF-1 constructs were from a previous study (15, 14). The oligonucleotides were radiolabeled by γ-[35S]ATP and protein kinase, and incubated with 10 ng of the purified TTF-1 HD-GST fusion protein alone or in combination. Electrophoretic mobility shift assay was performed by following the procedures described previously (14, 15). As a negative control, GST protein was also incubated with the radiolabeled probes alone or in combination with TTF-1 HD-GST or RARαs DBD-GST.

**Chromatin Immunoprecipitation Assay of RARαs and TTF-1 in H441 Cells**—The assay was performed using chromatin isolated from H441 cells. The following day, cells were treated with 10 µl of all trans retinoic acid for 24 h. Untreated cells served as controls. Cells were then treated with 1% formaldehyde in serum-free RPMI for 10 min at room temperature to cross-link proteins and DNA, followed by rinsing with cold PBS twice. The cell pellets were collected by centrifugation. The cells were lysed by adding 150 µl of lysis buffer (25 mM Tris, pH 8.1, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 3 mM EDTA, and 1X protease inhibitor mixture (Roche Molecular Biochemicals) and were allowed to incubate on ice for 10 min. The cells were sonicated followed by centrifugation, and the supernatants containing soluble chromatin were collected at 4 °C. The sonicated salmon sperm DNA (5 µg) was also added to cell lysate-antibody complex. Fifty µl of 50% protein A/G-agarose beads (Santa Cruz Biotechnology) were added to the samples and incubated at 4 °C for 2 h followed by centrifugation. The pellets were washed three times, once by 100 µl of 150 mM NaCl buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), once by 100 µl of TSE 500 mM NaCl buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 500 mM NaCl) and once by 100 µl of Buffer III (0.25 mM LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.1). Each wash was performed on ice for 10 min. Next, the samples were washed three times with 100 µl of TSE buffer. The pellets were then eluted off the beads by incubation with 1% SDS and 0.1 M NaHCO3 on ice for 10 minutes. Samples were heated at 65 °C for 4 h to reverse formaldehyde cross-linking followed by phenol-chloroform extraction and sodium acetate/ethanol precipitation. The DNA was then used as templates for quantitative PCR analysis with primers corresponding to the SP-B enhancer region.

**RESULTS**

**Co-localization of RARαs and TTF-1 in H441 Cells by Immunofluorescent Double Staining Assay**—To study whether RARαs and TTF-1 interact with each other to regulate hSP-B gene expression, RARαs and TTF-1 colocalization was assessed in H441 cells by double immunofluorescent staining. The hRARαs and TTF-1-FLAG expression vectors were cotransfected into H441 cells. Expression of TTF-1-FLAG protein (fluorescein isothiocyanate) and hRARαs protein (Texas red) was monitored by confocal microscope analysis of fluorescent image revealed colocalization of RARαs and TTF-1-FLAG proteins in the nucleus of H441 cells (Fig. 1).

**Synergistic Stimulation of the hSP-B500 by RARαs and TTF-1**—Overlapping of clustered RARαs and TTF-1 DNA binding sites located in the 5′-flanking region of the hSP-B gene provides the possibility that two proteins may interact with each other to synergistically stimulate hSP-B promoter expression. RARαs and TTF-1 expression vectors were cotransfected with the hSP-B500 luciferase reporter gene into H441 cells. The stimulatory effect of double transfection of RARαs and...
TTF-1 was much higher than the additive effect of RARα and TTF-1 transfection alone (Fig. 2), indicating that RARα and TTF-1 may interact to stimulate the hSP-B promoter in respiratory epithelial cells.

Protein-Protein Interaction between RARα and TTF-1 by GST Pull-down Study—To prove a direct interaction between RARα and TTF-1, a GST pull-down assay was performed. RARα was fused with GST to make a fusion protein. The purified RARα-GST fusion protein was incubated with the [35S]methionine labeled TTF-1 protein. After purification of incubated protein complexes through a Sepharose 4B-glutathione column, the radiolabeled TTF-1 protein was retained by the RARα-GST fusion protein as detected by polyacrylamide gel electrophoresis and autoradiography (Fig. 3). The GST control alone showed a very weak nonspecific pull-down signal, whereas the empty vector control (PCR3.0) showed no signal at all, indicating that [35S]Met-TTF-1 pull-down by the RARα-GST fusion protein was specific. This indicates the direct protein-protein interaction between RARα and TTF-1 in vitro. The interaction between RARα and TTF-1 was not further enhanced by RA treatment (data not shown), suggesting that the ligand-dependent AF-2 domain may not be required for interaction with TTF-1.

TTF-1 HD Interaction with RARα—To better understand the mechanism by which RARα and TTF-1 interact with each other, specific domains of TTF-1 required for RARα interaction need to be defined. Previously, TTF-1 functional domains were characterized (Fig. 4A) (22). The N- and C-terminal domains of TTF-1 were transactivation domains for hSP-B promoter activation. The HD of TTF-1 was the DNA binding and nuclear localization domain. Different portions of the TTF-1 molecule were constructed and radiolabeled with [35S]methionine. The radiolabeled TTF-1 fragments were incubated with the full-length RARα-GST fusion protein for pull-down. TTF-1 N-terminal and C-terminal domains failed to be pulled down by RARα-GST, whereas the TTF-1 HD was successfully pulled down by RARα-GST (Fig. 4B). Other TTF-1 fragments containing the HD were also pulled down by RARα-GST. Therefore, the TTF-1 HD is responsible for protein-protein interaction with RARα. The interaction appears to be enhanced by the N-terminal domain of TTF-1.

RARα DBD Interaction with TTF-1 HD—RARα is composed of several functional domains, including AF-2, DBD, LBD, and AF-1 domains (1). Because RAR and TTF-1 DNA binding sites overlap in the enhancer region of the hSP-B gene, it is highly likely that RARα DBD is involved in the protein-protein interaction with TTF-1. The AF-2 domain is required for ligand-dependent pull-down of nuclear receptor coactivators (25, 29–31). Therefore, both RARα DBD and AF-2 domains were selected for further study with TTF-1. Different portions of the TTF-1 molecule were radiolabeled with [35S]methionine. The radiolabeled TTF-1 fragments were incubated with the purified RARα-DBD-GST or RARα-AF2-GST fusion proteins for pull-down. The full-length TTF-1 and the TTF-1 HD were pulled down by RARα-DBD GST, but not by RARα-AF2-GST (Fig. 5). This is in agreement with the observation that TTF-1 pull-down by RARα was not RA-dependent. Therefore, DNA binding domains for both proteins are involved in protein-protein interaction in vitro.

Protein-Protein Interaction between RARα and TTF-1 in the Mammalian Two Hybrid System—To confirm that RARα DBD and TTF-1 HD are required for the interaction with partners in cells, a mammalian two hybrid system was used. The pair of TTF-1 HD AD/RARα BD constructs (Fig. 6A) and the pair of RARα DBD AD/TTF-1 BD constructs (Fig. 6B) were co-transfected into H441 cells with the luciferase reporter construct pG5LUC. The luciferase activities were markedly increased in paired cotransfection (Fig. 6). These findings further support the concept that DNA binding domains of both RARα and TTF-1 are required for protein-protein interaction both in vitro and in cells.

RARα DBD Effect on TTF-1 HD DNA Binding Affinity—The protein-protein interaction between DNA binding domains of RARα and TTF-1 prompted us to exam how this interaction alters their DNA binding affinity in the enhancer region of the hSP-B gene. Because the HD is the DNA binding domain and is
sufficient for TTF-1 binding to the enhancer region of the hSP-B gene (14), the TTF-1 HD-GST fusion protein was used for the DNA binding assay. The purified TTF-1 HD-GST and RARα DBD-GST fusion proteins were incubated with the oligo Ba (–439 to –410) from the enhancer region of the hSP-B gene, which contains overlapping RARE and TTF-1 DNA binding sites as reported previously (15), individually or in combination. The DNA-protein interactions were monitored by electrophoretic mobility shift assay. The RARα DBD-GST fusion protein alone had no detectable DNA binding activity to oligo Ba. This is in agreement with previous observations that DNA binding activity of RARα required dimerization with RXR. Interestingly, the RARα DBD-GST fusion protein significantly enhanced the DNA binding affinity of the TTF-1 HD-GST fusion protein.
plexes were separated on 4% nondenaturing polyacrylamide gels. Ar-
rows indicate the TTF-1-HD-GST DNA complex. The core sequence for
TFF-1 binding is underlined, and that for RARE is italicized.

RA Enhances RAR and TTF-1 DNA Binding to the hSP-B 5'-Flanking Regulatory Region in H441 Cells—In vivo chromatin immunoprecipitation assay was used to test whether RA treatment enhances RAR and TTF-1 binding affinity to the 5'-flanking regulatory region of the hSP-B gene in cells. The primers corresponding to hSP-B base pair –500 to +41 fragment, which contains both clustered RAR and TTF-1 sites, were used for PCR of immunoprecipitated chromatin. The monolayer of H441 cells was treated with all-trans RA (10^{-5} M). The untreated cells were used as a control. After protein-DNA cross-linking, soluble chromatin of H441 cells was prepared by analytic PCR using paired primers corresponding to the hSP-B –500 to +41 fragment. RA treatment did not generate the hSP-B promoter in the enhancer region of the hSP-B gene (15). Both RAR and TTF-1 were expressed in H441 cells and stimulated the hSP-B promoter in dose-dependent fashions (7, 15). After separation from the downstream TTF-1 sites, the clustered RARE sites in the enhancer region still rendered the SV40 promoter response to stimulation by RA (15). Therefore, the enhancer of the hSP-B gene works as an independent unit in which the tissue-specific factor TTF-1 determines RA/RAR signaling activity in pulmonary epithelial cells.

To determine whether RAR and TTF-1 directly interact with each other, protein-protein interaction studies were performed in the present study. There are three isotypes of RAR mediat-
ing RA function in cells. Only RARα was detected both in mouse type II epithelial cells and in the H441 cell line that shares characteristics of non-ciliated bronchiolar cells (7, 15). Therefore, RARα was chosen for protein-protein interaction with TTF-1 in H441 cells. GST pull-down experiments demonstrated the direct protein-protein interaction between the two proteins (Fig. 3). Deletion/truncation studies further defined that the DBD domain of RARα and the HD of TTF-1 were required for protein-protein interaction (Figs. 4–6). Interestingly, the DBD domain of RARα enhanced TTF-1 HD DNA binding affinity on an hSP-B enhancer oligonucleotide sharing the overlapping RARE/TTF-1 binding sites (Fig. 7). This may result from conformational changes of the oligonucleotide or TTF-1 in the presence of RARα BD. This process seems not be affected by RA. The ligand-dependent AF-2 domain, which is required for recruiting nuclear receptor coactivators through protein-protein interaction, was not required for protein-protein interaction with TTF-1. In addition, RAR and TTF-1 were colocalized in the nucleus of H441 cells as demonstrated by confocal/double immunofluorescent study (Fig. 1). Chromatin immunoprecipitation study showed that RA treatment increased recruitment of RAR and TTF-1 proteins to the enhancer region of the endogenous hSP-B gene in H441 cells (Fig. 8). Collectively, our data support the idea that RA/RAR

Pulmonary surfactant is synthesized and secreted primarily by type II epithelial cells in the alveoli of the lung. Deficiency or disruption of pulmonary surfactant causes respiratory distress syndrome. Surfactant proteins facilitate the spreading and enhance the stability of phospholipids in the alveoli and play an important role in host defense. Transcriptional regulation of surfactant protein genes by hormones and tissue-specific tran-
scription factors is the key step for elucidation of surfactant homeostasis in lung development and postnatal respiratory adaptation.

RA was shown to be important for the stimulation of hSP-B gene expression at the transcriptional level. Although the RA/RAR signaling pathway is well known to be critical to epithelial cell differentiation and proliferation in many tissues, little is known about how this pathway interacts with and is determined by tissue-specific factors in the respiratory system. We previously demonstrated that in the pulmonary epithelial system, RA stimulation of the hSP-B promoter through RARE sites is dependent on the juxtaposed clustered TTF-1 sites in the enhancer region of the hSP-B gene (15). Both RARα and TTF-1 were expressed in H441 cells and stimulated the hSP-B promoter in dose-dependent fashions (7, 15). After separation from the downstream TTF-1 sites, the clustered RARE sites in the enhancer region still rendered the SV40 promoter response to stimulation by RA (15). Therefore, the enhancer of the hSP-B gene works as an independent unit in which the tissue-specific factor TTF-1 determines RA/RAR signaling activity in pulmonary epithelial cells.

DISCUSSION

Pulmonary surfactant is synthesized and secreted primarily by type II epithelial cells in the alveoli of the lung. Deficiency or disruption of pulmonary surfactant causes respiratory distress syndrome. Surfactant proteins facilitate the spreading and enhance the stability of phospholipids in the alveoli and play an important role in host defense. Transcriptional regulation of surfactant protein genes by hormones and tissue-specific tran-

FIG. 7. Enhancement of TTF-1 HD DBD binding affinity by RARα DBD. The radiolabeled oligonucleotide Ba probe was incubated with the purified TTF-1 HD-GST fusion protein or the RARα DBD GST fusion protein or in combination. Free probes and DNA-protein complexes were separated on 4% nondenaturing polyacrylamide gels. Ar-
rows indicate the TTF-1-HD-GST DNA complex. The core sequence for
TFF-1 binding is underlined, and that for RARE is italicized.

FIG. 8. Chromatin immunoprecipitation assay of RAR and TTF-1 DNA binding to the hSP-B 500 enhancer in vivo. H441 cells were harvested with or without RA (10^{-5} M) treatment. Soluble chromatin was immunoprecipitated with RAR antibody (A) or TTF-1 antibody (B) and FLAG antibody (C). Coprecipitated DNA was analyzed by PCR using a pair of primers corresponding to the hSP-B promoter/ enhancer region (base pairs –500 to +41). In C, column C is a positive control and represents PCR products using the hSP-B 500 plasmid as a template (control). MW, molecular weight.
not only depends on TTF-1 to stimulate the hSP-B promoter but also facilitates TTF-1 binding to the hSP-B enhancer region. TTF-1 and RAR together synergistically stimulate hSP-B transcription.

The complexity of RAR stimulation on target genes depends on multiple protein factors interacting with its various transcriptional domains, including transcriptional intermediary factor 2 (24, 25), AP-1 (26), TFIH (27), and TAFI135 (28), among others. Most importantly, RAR recruits p160 nuclear receptor coactivators and CBP/p300 in the presence of RA through physical interaction with the AF-2 domain (25, 29–31). We previously demonstrated that CBP and p160 nuclear receptor coactivators (steroid receptor coactivator 1, transcriptional intermediary factor 2, and activator of thyroid and retinoic acid receptor) significantly stimulated the hSP-B promoter in a dose-dependent fashion (15). p160 nuclear receptor coactivators and CBP interacted with TTF-1 in the mammalian two hybrid system and synergistically stimulated hSP-B500 with TTF-1 (19). They were all colocalized with SP-B in developing and adult epithelial cells in the lung (19). Coiling of DNA around a histone octamer in the nucleosome is a cornerstone of transcriptional control. Nucleosomes repress all genes, including genes essential for respiratory functions. They occlude sites of protein binding to DNA and interfere with the interaction of activators, polymerases, transcription factors, and DNA-modifying enzymes (32). Therefore, relief of repression by chromatin is the first step toward the initiation of gene transcription. p160 nuclear receptor coactivators and CBP/p300 possess intrinsic histone acetyltransferase activity, which reversibly acetylates specific lysine residues within the N-terminal tails of core histones and leads to chromatin remodeling and gene activation (32).

Based on our findings, an enhanceosome model was postulated that TTF-1, RAR/RXR, CBP, and p160 coactivators form a transcriptional complex in the enhancer region of the hSP-B gene (19), which may play an important role in temporal and spatial expression of SP-B during lung development. The interaction between RAR and TTF-1 provides a foundation for the enhanceosome formation in the enhancer region of the hSP-B gene. Because both RAR and TTF-1 are essential for lung organogenesis and branching morphogenesis, our study provided direct evidence and a model system to explain how RAR and TTF-1 interact with and depend on each other to regulate lung-specific gene expression at the transcriptional level to influence lung development.

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