THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 17, Issue of April 23, pp. 17384–17390, 2004
Printed in U.S.A.

TAZ Interacts with TTF-1 and Regulates Expression of Surfactant Protein-C*

Received for publication, November 17, 2003, and in revised form, February 13, 2004
Published, JBC Papers in Press, February 17, 2004, DOI 10.1074/jbc.M312569200

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Thyroid transcription factor-1 (TTF-1/Nkx-2.1) is required for formation of the lung and differentiation of peripheral respiratory epithelial cells. TTF-1 activates transcription of target genes, including the surfactant proteins critical for lung function. A recently identified protein TAZ (transcriptional co-activator with PDZ-binding motif) contains a WW domain and a COOH-terminal PDZ-binding motif that are proposed to mediate its interactions with various transcriptional proteins. To determine the role of TAZ in the regulation of gene expression in the lung, the sites of TAZ expression and the role of TAZ in the regulation of respiratory epithelial gene expression were assessed. TAZ mRNA was detected in immortalized mouse lung epithelial cells, primary isolates of mouse alveolar type II epithelial cells, and epithelial cells of fetal lung. Sites of TAZ mRNA and protein overlapped with those of TTF-1 and surfactant protein C (SP-C) in the respiratory epithelial cells of the mouse lung. In the presence of TTF-1, TAZ synergistically activated the expression of mouse SP-C-luciferase reporter constructs. Mammalian two-hybrid assays and pull-down experiments demonstrated that the TAZ directly interacted with TTF-1. Further, deletion analysis demonstrated that TAZ binds to the NH_2-terminal domain of TTF-1. TAZ binds to TTF-1, increasing the transcriptional activity of TTF-1 on the SP-C promoter. Developmental and cell-selective regulation of TAZ provides a mechanism by which the activity of TTF-1 on target genes is modulated.

Thyroid transcription factor-1 (TTF-1,[1] also termed T-EBP/Nkx2.1) is a member of the Nkx-2 class of homeodomain-containing transcription factors (1, 2) that is selectively expressed in the developing thyroid, respiratory epithelium, and restricted areas of the developing brain (3–5). TTF-1 expression begins at the onset of thyroid and lung formation, and its expression is maintained in a highly regulated pattern in the thyroid follicular cells and subsets of respiratory epithelial cells (6–8). In the thyroid, TTF-1 regulates thyroid-specific genes including thyroglobulin, thyroperoxidase, and the sodium-iodide symporter (1, 9, 10). In the lung, TTF-1 activates the expression of surfactant proteins A, B, and C (SP-A, SP-B, and SP-C) and Clara cell secretory protein (11–16). The critical role of TTF-1 for lung morphogenesis was shown in the study of TTF-1-null mice that lacked lung parenchyma, thyroid, and pituitary glands. TTF-null mice also have defects in the ventral area of the forebrain (4, 5). Clinically, mutations in the human *Ttf-1* gene result in central nervous system, thyroid, and respiratory disorders. Human TTF-1 haploinsufficiency has been linked to choreoathetosis, hypothyroidism, and various pulmonary disorders including neonatal respiratory distress and pulmonary infections (17). Transgenic mice in which TTF-1 is overexpressed in alveolar type II cells caused dose-dependent epithelial cell hyperplasia, emphysema, and pulmonary inflammation (18). Taken together, the temporal, spatial, and quantitative regulation of TTF-1 activity is critical for normal lung morphogenesis and physiology. TTF-1 binds an element termed the NKX binding element(s), containing 5′-TNAAGTG-3′, found in regulatory regions of target genes (19). Multiple NKX binding element(s) have been identified in the promoters of surfactant protein and Clara cell secretory protein genes (12, 20). These elements are required for the activity of the surfactant promoters. TTF-1 directly activated the promoters of target genes when expressed in non-respiratory epithelial cells. However, the mechanisms by which TTF-1 regulates transcriptional activity on target promoters remain to be defined. TTF-1 contains three distinct domains: an NH_2-terminal transactivation domain, a DNA-binding homeodomain, and a COOH-terminal activation domain. Structural and functional studies of TTF-1 NH_2-terminal domain have suggested that the NH_2-terminal domain has functional properties similar to the typical acidic activation domain in VP16 (20, 21). The NH_2-terminal domain was shown to mediate the primary transactivation activity because a TTF-1 mutant in which the NH_2-terminal domain was deleted lost its transcriptional activity, whereas a mutant in which the COOH terminus of TTF-1 was deleted partially reduced activity (22). Differences between the NH_2-terminal and COOH-terminal activation domains of TTF-1 were not observed when tested in the thyroglobulin promoter (20, 23). Thus, the NH_2- and COOH-terminal domains may mediate interactions of TTF-1 with other proteins to regulate target gene expression in a gene-specific manner.

Consistent with this concept, it is increasingly clear that TTF-1 functions cooperatively with a number of other tran-

* This work was supported by National Institutes of Health Grants HL 38859 and HL 61646 (to J. A. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TTF-1, thyroid transcription factor-1; MLE, mouse lung epithelial; SP-A, SP-B, SP-C, surfactant protein A, B, C; NFI, nuclear factor-I; AEII, alveolar type II; RT, reverse transcription; CAT, chloramphenicol acetyltransferase; GST, glutathione *S*-transferase; GFP, green fluorescent protein; EGFP, enhanced GFP; luc, luciferase; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; C/EBP, CCAAT/enhancer-binding protein; E, embryonic day.

scription factors, forming complexes on regulatory regions of target genes. TTF-1 interacts with Pax8, GATA6, STAT3, retinoic acid receptor and associated cofactors, nuclear factor-I (NFI-B), AP1 family members, and BR22 (23–29).

TAZ (transcriptional co-activator with PDZ-binding motif) was recently discovered as a 14-3-3-binding molecule (30). Most human and mouse tissues, except thymus and peripheral blood leukocytes, express TAZ mRNA. The highest levels of expression were observed in kidney, heart, and lung. TAZ contains a central WW, a COOH-terminal sequence that contains a region predicted to form a two-stranded coiled-coil domain, and a PDZ-binding motif at the COOH terminus that localizes TAZ into discrete nuclear foci. TAZ functions as a transcriptional co-activator through binding of the WW domain to the (L/P)P*X*Y amino acid motif present on transcription factors. Interactions with 14-3-3 and an unknown PDZ domain protein may therefore regulate trafficking or transcriptional activities of binding partners (30–33). The presence of (L/P)P*X*Y motif in TTF-1 provides a potential interaction with TAZ. Although readily detected in lung, TAZ mRNA was not detected in thyroid, a site of TTF-1 activity.

In the present study, we have shown that TAZ, TTF-1 and SP-C are co-expressed in epithelial cells of the fetal lung. TAZ physically interacted with TTF-1 through WW domain binding to an NH_2-terminal LPPY motif of TTF-1 to regulate TTF-1-mediated activation of the gene encoding SP-C (*sftp-c*) in respiratory epithelial cells.

EXPERIMENTAL PROCEDURES

Isolation of RNA from Mouse Embryos and Cells, and Reverse Transcription (RT)-PCR—Lungs from fetal mice were obtained at E11.5, 12.5, 13.5, 15.5, 16.5, and 17.0 and lysed for RNA preparation. MLE-15 and HeLa cells were lysed in Trizol reagent, and total RNA was extracted according to the manufacturer's specifications (Invitrogen). The final RNA pellet was dissolved in diethylpyrocarbonate-treated water. RNA from purified mouse alveolar type II cells (AEII) was kindly provided by Dr. Timothy Weaver (Cincinnati Children's Hospital, Cincinnati, OH), and AEII cells were prepared from 6-week-old female C57B/6 mice as described previously (34). RNA was reverse-transcribed, and 1/20 volume of reverse-transcribed product was used for subsequent PCR using procedures provided in a kit provided by the manufacturer (Invitrogen). The PCR primers used were as follows: *TAZ* 5′ primer, 5′-CGT CCA TCA CTT CCA CCT CG-3′; 3′ primer, 5′-ACT GTA GCA CCC TAA CCC CAG G-3′; *β-actin* 5′ primer, 5′-GTC CCT GTA TGC CTC TGG TC-3′; 3′ primer, 5′-TCG TAC TCC TGC TTG CTG AT-3′. The PCR conditions were as follows: 95 °C for 5 min for 1 cycle, 30 cycles of 95 °C for 1 min, annealing at various temperatures for 30 s, 72 °C for 30–40 s, with a final extension cycle of 72 °C for 7 min. The annealing temperatures were as follows: *TAZ,* 58 °C; *β-actin,* 60 °C. The PCR products were resolved by gel electrophoresis on 1% agarose gels containing ethidium bromide.

In Situ Hybridization—Whole mount *in situ* hybridization was performed with digoxigenin-labeled antisense RNA probes essentially as described previously (35). Mouse fetal lungs at embryonic days 11.5, 12.5, and 13.5 were dissected and fixed with 4% paraformaldehyde/phosphate-buffered saline. The template for making antisense and sense RNA probes was generated by amplifying a part of TAZ cDNA (GenBank™ accession number AJ299430). The 5′ primers for the PCR have T3 promoter sequence at the 5′ end and 3′ primer T7 promoter at the 3′ end, and the sequences are as follows: 5′ primer, 5′-**AAT TAA CCC TCA CTA AAG GG** CAC CAA CAC CAG CAA GTC GTG-3′, and 3′ primer, 5′-**TAA TAC GAC TCA CTA TAG GG** AGG AAA TCA GGG AAG CGG G-3′. (The sequences of T3 and T7 promoters are written in bold.) The antisense probe was transcribed using T7 promoter and the sense probe was transcribed using T3 promoter according to procedures provided in Mega kit for in vitro transcription (Ambion). Hybridization was performed overnight at 70 °C in the solution of 50% formamide, 5× SSC, 1% SDS, 50 μg/ml yeast tRNA, 50 μg/ml heparin sodium in diethylpyrocarbonate-H_2O. *In situ* hybridization on sections was performed with ^{35}S-nucleotide labeled (1,000 Ci/nmol, Amersham Biosciences) riboprobes to assess the sites of expression of TAZ mRNA. Sense and antisense riboprobes were generated from the same template as used above using a Riboprobe transcription kit (Promega, Madison, WI). Conditions and solutions for hybridization are essentially as described previously (36). Slides were dipped in KodakNTB2 emulsion, exposed for 1–6 weeks, and developed with Kodak D19 developer following the manufacturer's protocols. After development, sections were examined and photographed under phase optic and dark field illumination.

Immunohistochemistry—Mouse fetal lung tissues were obtained at different developmental stages and then processed according to a standard method (18) and embedded in paraffin. Immunostaining of TAZ was performed essentially as described previously (8). Goat polyclonal antibody against amino acids of TAZ (Santa Cruz Biotechnology, Santa Cruz, CA; sc-17230) was used at a dilution of 1:25 after blocking tissue sections with 4% horse serum in phosphate-buffered saline/0.2% Triton X-100.

Plasmids—pCMV-TAZ was generated by PCR amplification of mouse TAZ coding region and subsequent cloning in the EcoRI-MluI sites of the pCMV5 vector (37). pEGFP-TAZ, pEGFP-TAZ/S89A, and pEGFP-TAZ/-4 were produced previously (30). The GST·TAZ fusion protein was generated by PCR amplification of TAZ coding region and subsequent subcloning in the EcoRI-XhoI sites of the pGEX-4T3 vector. The correct frame of the fusion was verified by DNA sequencing. 3xFLAG-TTF-1 was generated by PCR amplification of TTF-1 coding region and subsequent cloning in the EcoRV-BamHI sites of the 3xFLAG CMV-10 vector (Sigma). 3xFLAG-Δ3 was generated by PCR amplification of the coding region of TTF-1 deletion mutant Δ3 and subcloned in the NotI-XbaI sites of the 3xFLAG CMV-10 vector (Sigma). The plasmids used in the transient transfection experiments have been described previously and were as follows: CMV-TTF-1 (12), Δ14, and Δ3 (20). Reporter constructs containing the luciferase gene and the 4.8- and 0.32-kb DNA fragments of the 5′-flanking region of the mouse *SP-C* promoter (4.8 mSP-C-luc and 0.32 mSP-C-luc, respectively) were made previously (14).

Cell Culture and Reporter Assays—MLE-15 cells are cloned SV40 large T antigen immortalized mouse lung epithelial cells that express endogenous TTF-1 and surfactant proteins (39). MLE-15 cells were cultured in HITES medium (RPMI 1640 containing 10 mM HEPES, 5 μg/ml insulin, 10 μg/ml transferrin, 3×10^{-8} M sodium selenite, 1×10^{-8} M β-estradiol, and 200 mM L-glutamine) and were seeded at 40–50% confluence in 6-well plates for transfection. HeLa cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum. For transactivation experiments, HeLa cells were seeded at a density of 2×10^5 cells/well in 6-well plates. The reporter constructs 4.8 mSP-C-luc or 0.32 mSP-C-luc were co-transfected with empty vector, expression vectors encoding transactivators (TAZ, TTF-1, and their variants; see "Plasmids"), or 0.5 μg of pCMV-β-galactosidase using FuGENE 6 (Roche Applied Science). The total amount of transfected DNA was fixed by adding the corresponding amount of empty vectors. After 48 h of incubation, lysates were assayed for β-galactosidase and luciferase activity (Promega, Madison, WI). Light units were assayed by luminometry (Monolight 3010, Analytical Luminescence Laboratory, San Diego, CA). Relative light units were normalized to β-galactosidase activity.

Levels of TAZ and mutant TAZ proteins produced after transfection were measured by immunoblot analysis using HeLa cell lysates. Total protein from cell lysates was resolved on 10–20% SDS-PAGE and transferred to nitrocellulose membrane. Blots were incubated with goat anti-GFP antibody (1:100, Santa Cruz Biotechnology) for EGFP-tagged TAZ and its variants and monoclonal anti-TTF-1 Ab-1 (clone 8G7G3/1, Neo Markers, Lab Vision Corp.) for TTF-1 and its variants.

Mammalian Two-hybrid System Assays—The reporters pG5Luc and pM/TTF-1 were kind gifts from Dr. Cong Yan, (Cincinnati Children's Hospital, Cincinnati, OH). TAZ cDNA encoding the full length was cut with EcoRI and subcloned into pVP16 (Clontech) at the EcoRI site. Transfection assays using MLE-15 cells were performed as described previously (38).

In Vitro Protein Interaction—GST·TAZ protein was purified from BL21 (DE) LysS bacterial cells transformed with pGEX-TAZ. At A_{600} = 0.6, isopropyl-1-thio-β-D-galactopyranoside (0.1 mM final) was added to the culture to induce the expression of the fusion protein, and cells were resuspended in lysis buffer (phosphate-buffered saline, 0.5 mM EDTA, 1 mg/ml lysozyme, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, protease inhibitors diluted 1:1000) and sonicated. Triton X-100 was added to 1%, and the cell extract was centrifuged at 40,000 rpm for 40 min at 4 °C. The supernatant was then subjected to affinity chromatography using glutathione-Sepharose beads (Amersham Biosciences). After binding, beads were washed three times with buffer containing 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, and 5 mM dithiothreitol. GST·TAZ was eluted with a buffer containing 10 mM glutathione, 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, for 10 min at 4 °C.

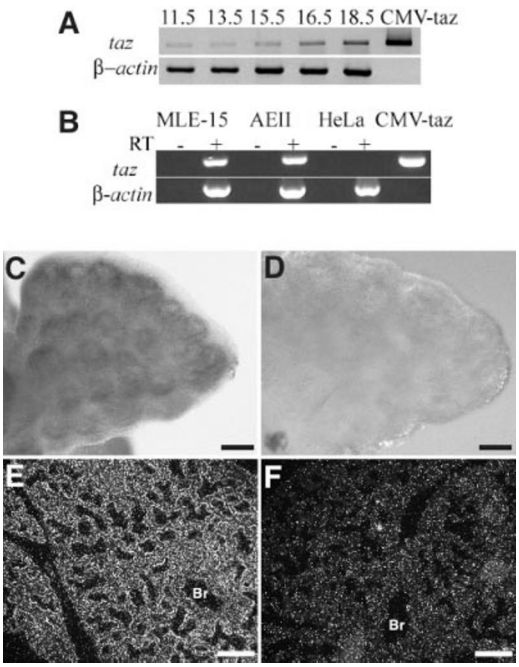

FIG. 1. **TAZ mRNA is expressed in the fetal lung and respiratory epithelial cells.** RT-PCR was performed using total RNA from mouse fetal lungs at E11.5, 13.5, 15.5, 16.5, and 18.0 (*A*) and from AEII, MLE-15 cells, and HeLa cells (*B*). TAZ mRNA was detected at all embryonic stages tested and in AEII and MLE-15 cells. A plasmid containing full-length TAZ cDNA was used for a positive control, and a *β-actin* mRNA fragment was amplified as control. *CMV*, cytomegalovirus. TAZ mRNA was detected in both epithelium and mesenchyme of the mouse fetal lung at E13.5 (*C*) and E18.0 (*E*). Hybridization was observed primarily in the respiratory epithelium of the peripheral lung and not in the conducting airways (*Br*, bronchiole). Lungs hybridized with sense probes showed no detectable signal (*D* and *F*). *Bars* = 100 μm.

The eluted protein was stored at −80 °C. Protein concentration was judged from Coomassie Blue staining. A pull-down assay was performed by incubating 4 μg of GST or GST·TAZ-purified proteins bound previously to glutathione-agarose beads with 3 mg of total protein extract prepared from HeLa cells. The binding reactions were carried out for 90 min at 4 °C on a rotating wheel. Beads were washed several times with a buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Triton x-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (1:1000) (Sigma). The bound proteins were eluted by resuspending the beads directly in 2× SDS-PAGE sample buffer and heating at 95 °C for 3–5 min before loading on the gel.

RESULTS

TAZ Is Expressed in Respiratory Epithelial Cells of the Developing Lung—Since their structures indicate the potential for interactions between TAZ and TTF-1 in the regulation of surfactant gene expression, it was relevant to test whether the two proteins co-exist in respiratory epithelial cells. To determine whether the temporal/spatial expression pattern of TAZ in the developing mouse lung is consistent with that of TTF-1, RT-PCR, *in situ* hybridization, and immunohistochemical studies were performed. RT-PCR was conducted using total RNA from mouse fetal lungs obtained on E11.5, 13.5, 15.5, 16.5, and 18.0. TAZ mRNA was detected at all ages tested, and the level of TAZ mRNA gradually increased with advancing gestation (Fig. 1*A*). TAZ mRNA was also detected at relatively high levels in purified mouse alveolar type II cells (Fig. 1*B*), which are known to express TTF-1 and surfactant proteins (7, 8, 24). The pattern of expression of TAZ mRNA in the lung was assessed by *in situ* hybridization from E11.5–18.0. Although TAZ mRNA was first detected in the lung buds at E12.5 (data not shown), the expression in the epithelium was more distinct at E13.5 (Fig. 1*C*). Although TAZ mRNA was detected in the respiratory epithelium, a diffuse signal was also detected in pulmonary mesenchyme. At E18.0, TAZ mRNA was detected primarily in the respiratory epithelium of peripheral lungs and was not detected in the conducting airways (Fig. 1*E*). Immunohistochemistry was used to identify sites of the TAZ protein in the developing mouse lung. TAZ staining was detected primarily in the epithelium of the fetal lungs (Fig. 2, *B–D*).

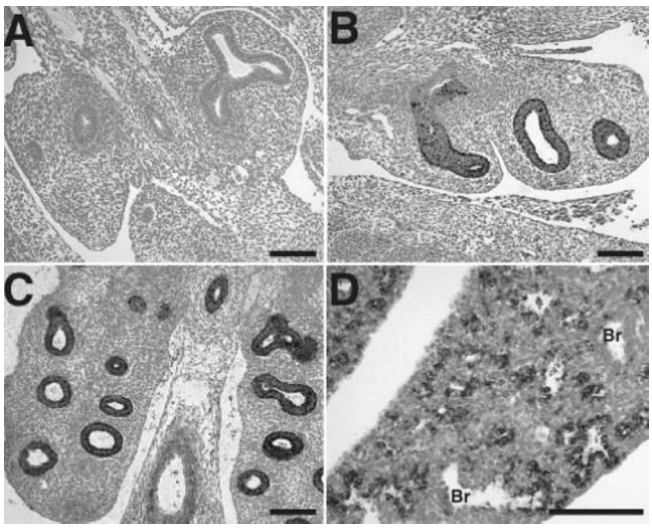

FIG. 2. **Immunohistochemical detection of TAZ in fetal mouse lung.** Paraffin sections of fetal mouse lungs were immunostained with goat polyclonal antibody against TAZ. TAZ was detected mainly in the fetal lung epithelium at E12.5 (*B*) and E13.5 (*C*) and in the respiratory epithelial cells at E17.5 (*D*) and not in the conducting airways (*Br*, bronchioles). A E12.5 lung section stained without the primary antibody showed no detectable signal (*A*). *Bars* = 100 μm.

TAZ and TTF-1 Synergistically Activate Transcription from the Mouse SP-C Promoter—To determine whether TAZ influences the activity of the SP-C promoter, TAZ alone or TAZ and TTF-1 were transiently co-transfected with a reporter plasmid (4.8 mSP-C-luc) consisting of 4.8 kb of the 5′ region of the mouse surfactant protein-C gene, *Sftp-c,* linked to a luciferase reporter gene. TAZ increased the activity of the mouse SP-C promoter and synergistically enhanced the effects of TTF-1 in MLE-15 cells (Fig. 3*A*). TAZ also activated a smaller SP-C promoter fragment (0.32 mSP-C), albeit to a lesser extent (Fig. 3*B*). Likewise, TAZ enhanced the activity of an artificial TTF-1-responsive promoter that consists of five consecutive binding sites for TTF-1 and controls expression of chloramphenicol acetyltransferase (CAT) (C5-CAT) in HeLa cells,[2] supporting its potential interaction with TTF-1. When MLE-15 cells were transfected with increasing amounts of TAZ and TTF-1, stimulatory effects of TAZ on the SP-C promoters were dose-dependent (Fig. 3*E*). Since TAZ and TTF-1 are expressed in MLE-15 cells, the activation by TAZ may represent co-activation. Two TAZ mutants were generated previously, substituting a serine residue in the 14-3-3-binding site to alanine (TAZ S89A) and deleting the last four COOH-terminal residues (TAZ-4) within the PDZ-binding motif, respectively (30). Previously, it was shown that TAZ S89A increased transcriptional activity by 1.5–2-fold as compared with wild type, whereas TAZ-4 lost the ability to co-activate the reporter gene (30). Increasing amounts of these TAZ mutants were transfected in MLE-15 cells. The active S89A TAZ mutant increased the transcriptional activity of both 4.8 and 0.32 mSP-C-luc reporters, whereas the inhibitory mutant, TAZ-4, blocked the effects of TAZ on TTF-1-dependent activity (Fig. 3*E*). Taken together,

[2] M. Zannini, unpublished results.

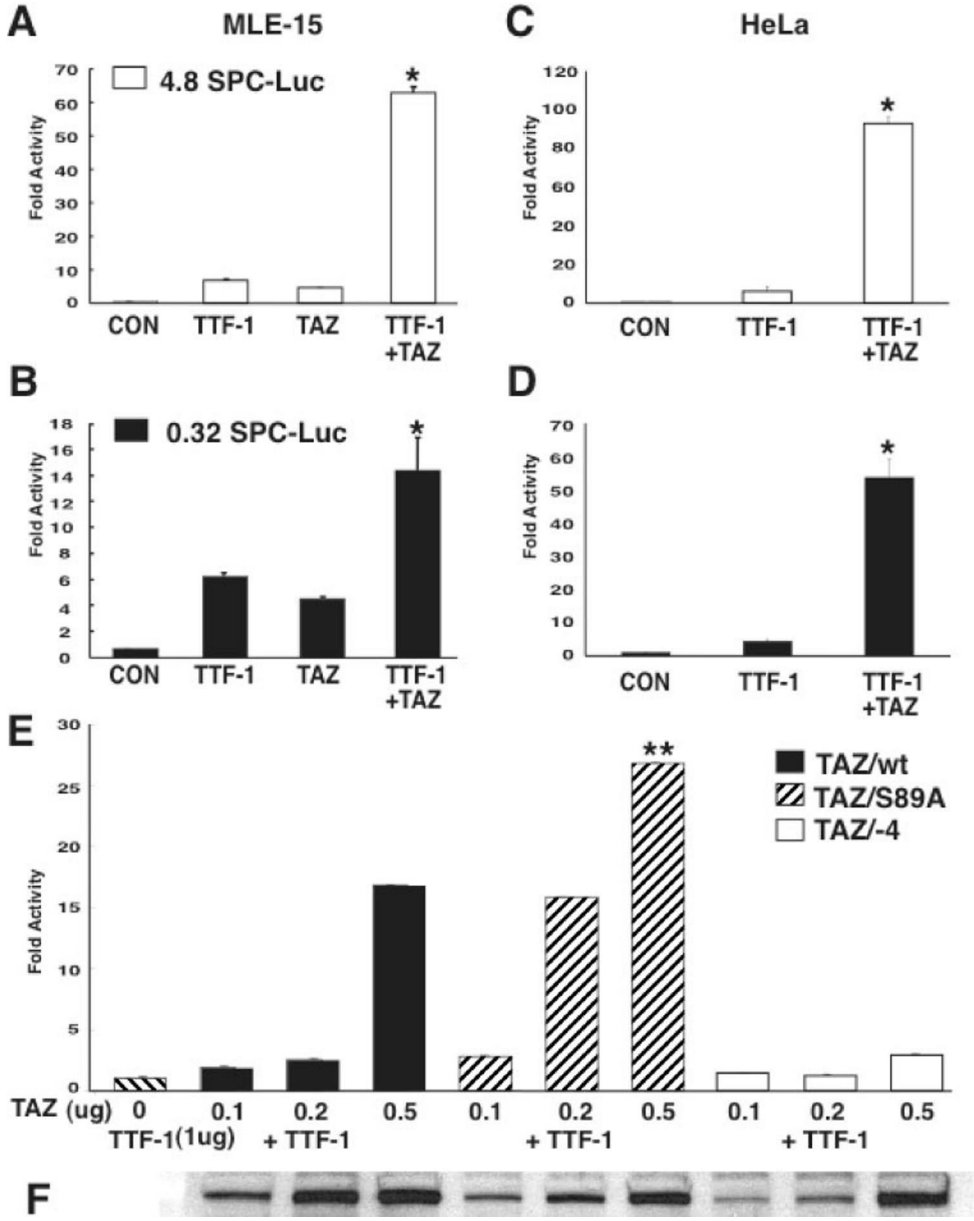

FIG. 3. **TAZ enhances TTF-1 activity on the SP-C promoter.** MLE-15 cells (*A*, *B*, and *E*) or HeLa cells (*C* and *D*) were co-transfected with expression vectors for TAZ and TTF-1 in the presence of reporter constructs (4.8 mSP-C-luc or 0.32 mSP-C-luc) containing a luciferase gene under control of 4.8 kb of the 5′-flanking region of mouse surfactant protein-C (4.8 mSP-C) (*A* and *C*) or 320 bp of SP-C promoter (0.32 mSP-C) (*B* and *D*). Cells were transfected with an expression vector for β-galactosidase as an internal control (*CON*). The total amount of transfected DNA was fixed by adding the corresponding amount of empty vector (pCDNA 1.1). Cells were harvested after 48 h, and luciferase and β-galactosidase activity were measured. Relative light units were normalized to β-galactosidase activity. The results were shown as -fold increases in activity as compared with the control (*A–D*, 4.8 mSP-C-luc or 0.32 mSP-C-luc alone). Values are mean ± S.D., $n = 3$. The experiments were repeated at least two times with similar results. *, $p < 0.001$ by analysis of variance as compared with the group transfected with TTF-1 expression vector. *E*, MLE-15 cells were transfected with increasing amounts of pEGFP-TAZ and the two mutants, pEGFP-TAZ S89A and pEGFP-TAZ-4. The stimulatory effects of TAZ and TAZ S89A were dose-dependent. The effects of TAZ S89A were higher than those of the TAZ wild type (*wt*). TAZ-4 inhibited the effects of TAZ on TTF-1-dependent activity. The results are presented as -fold increases in activity as compared with the control (0.32 mSP-C-luc+TTF-1). EGFP-tagged TAZ protein and variants were measured after inspection by immunoblot (*F*). Values are mean ± S.D., $n = 3$. **, $p < 0.001$ by analysis of variance.

TAZ-mediated transcriptional co-activation of the SP-C promoter involves binding of various PDZ domain-containing proteins. To test whether the effects of TAZ on transcriptional activity of TTF-1 is lung cell-specific, HeLa cells, which do not express TTF-1, were co-transfected with 4.8 or 0.32 mSP-C-luc reporters along with TAZ and TTF-1. TAZ markedly enhanced TTF-1 activity on the SP-C promoters (Fig. 3, *C* and *D*), although TAZ alone did not increase the activity of the reporter constructs in HeLa cells (data not shown). Taken together, the transcriptional co-activation effects of TAZ require interaction with TTF-1 on the SP-C promoters, supporting the concept that TAZ interacts with TTF-1 to modulate gene expression.

TAZ and TTF-1 Interact in Vitro—Since TAZ synergistically stimulated TTF-1 activity, we tested whether TAZ and TTF-1 directly interacted by a mammalian two-hybrid assay in MLE-15 and HeLa cells, as described previously (23, 38). Fusion genes (*vp16-taz* and *m-ttf-1*, Fig. 4*A*), encoding TAZ and the VP16 transactivation domain and a full-length TTF-1-Gal4-DNA-binding domain fusion protein, were co-transfected with the luciferase reporter construct containing five Gal4-binding sites (pG5-luc) to monitor protein-protein interactions between TAZ and TTF-1. Co-transfection of VP16-TAZ and

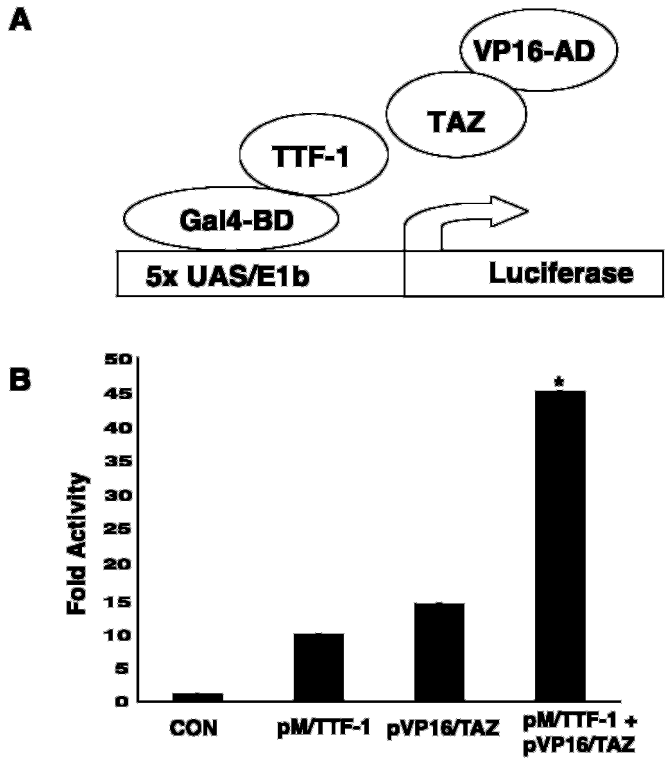

FIG. 4. **Interaction between TAZ and TTF-1 in the mammalian two-hybrid assay.** A model of the mammalian two-hybrid assay is represented (*A*). The luciferase reporter pG5Luc was co-transfected with the pairs of pM/TTF-1 and pVP16/TAZ into MLE-15 cells (1 μg of each) (*B*). Individual transfection controls (*CON*, pM + pVP16; pVP16/TAZ, pM + pVP16/TAZ; pM/TTF-1, pM/TTF-1 + pVP16) are shown. Values are mean ± S.D., $n = 3$. The experiment was repeated with a similar result. *, $p < 0.001$ by analysis of variance as compared with the group transfected with pM/TTF-1 and pVP16 (pM/TTF-1).

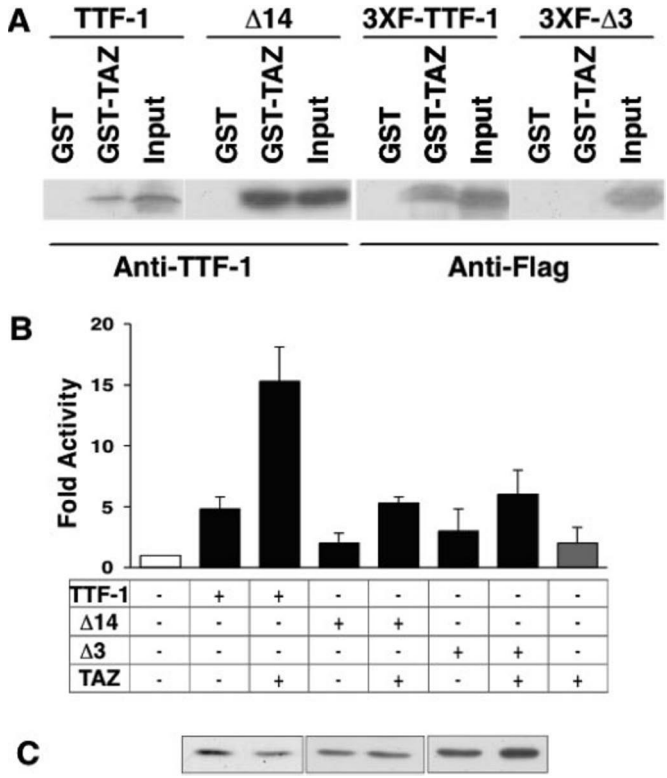

FIG. 5. **The TTF-1 NH_2-terminal domain is required for the physical but not for the functional interaction with TAZ.** GST pull-down assays were performed with GST·TAZ immobilized on glutathione-Sepharose beads and protein extract prepared from HeLa transiently transfected with the expression vectors encoding for TTF-1, Δ14, 3XF-TTF-1, and 3XF-Δ3 proteins. The extracts were incubated with GST or GST·TAZ. Both GST and GST·TAZ beads were washed several times before being boiled, run on 10% SDS-polyacrylamide gels, and analyzed by immunoblot using a specific polyclonal antibody against TTF-1 (for TTF-1 and Δ14) or a monoclonal antibody (Anti-FLAG M2 from Sigma) recognizing the FLAG epitope (for 3XF-TTF-1 and 3XF-Δ3) (*A*). HeLa cells were transiently transfected with the reporter plasmid 0.32 mSP-C-luc and the expression vectors encoding either wild-type TTF-1 or the deletion mutant Δ14 and Δ3, with or without co-transfection with TAZ. Fold of activation was considered as a ratio between values obtained with and without co-transfection of the expression vectors. Values are the mean of at least four independent experiments (*B*). The protein levels of TTF-1, Δ14, and Δ3 after transfection were measured by immunoblot and are shown (*C*).

M-TTF-1 markedly enhanced reporter gene expression, demonstrating a direct protein-protein interaction between TAZ and TTF-1 proteins in MLE-15 cells (Fig. 4*B*).

NH_2-terminal Domain of TTF-1 Is Required for Physical Interaction with TAZ but Not Sufficient for Functional Cooperation—To demonstrate the physical interaction between TTF-1 and TAZ, recombinant GST·TAZ fusion protein was purified from bacteria and used in pull-down assays with protein extracts from HeLa cells transiently transfected with an expression vector encoding TTF-1. The result of the pull-down experiment shows that GST·TAZ specifically binds wild-type TTF-1, whereas GST alone does not (Fig. 5*A*). To further map the domains of TTF-1-binding TAZ, we utilized pull-down assays. Pull-down experiments were performed using GST·TAZ fusion protein and protein extracts prepared from HeLa cells transiently transfected with the expression vectors encoding for NH_2-terminal and COOH-terminal TTF-1 mutants. In these experiments, constructs Δ14 and 3XFLAG-Δ3 encoding the NH_2-terminal domain and the COOH-terminal domain, were used (see "Experimental Procedures"). The 3XFLAG-Δ3 expression vector encodes for the COOH-terminal domain of TTF-1 fused to the FLAG epitope since the polyclonal antibody against TTF-1 that we used was generated against a peptide in the NH_2-terminal portion of the protein. Results of the binding reactions showed that TTF-1, Δ14, 3XFLAG-TTF-1 proteins were specifically bound by GST·TAZ, whereas the 3XFLAG-Δ3 protein was not, thus demonstrating that physical interaction between TAZ and TTF-1 was mediated through the NH_2-terminal domain of TTF-1 (Fig. 5*A*). To test whether the NH_2 terminus of TTF-1 was sufficient for the functional cooperation between TTF-1 and TAZ in the transcriptional activation of the SP-C promoter, we transfected HeLa cells with TTF-1 deletion mutants, designated Δ14 and Δ3 (Fig. 5*A*), and the reporter construct 0.32 mSP-C-luc and an expression vector encoding TAZ. The TTF-1 mutant Δ14, containing the NH_2-terminal activation domain and the homeodomain, was not able to significantly synergize with TAZ on the SP-C promoter as did wild-type TTF-1 (Fig. 5*B*). The TTF-1 mutant Δ3 did not significantly synergize with TAZ. Thus, the NH_2-terminal domain of TTF-1 is necessary for physical interaction with TAZ but is not sufficient for full synergism in the activation of the SP-C promoter.

DISCUSSION

The activity of transcription factors is often regulated by various co-regulators including co-activators, adapters, and co-repressors. Recently, a transcriptional co-activator TAZ was discovered and shown to interact with transcription factors, which contain (L/P)P*X*Y motif on their amino acid sequences. Although candidate nuclear factors have been proposed as putative TAZ-interacting proteins, few of them have been formally tested. In this study, we demonstrated that TAZ acts as a transcriptional co-activator of TTF-1, enhancing its tran-

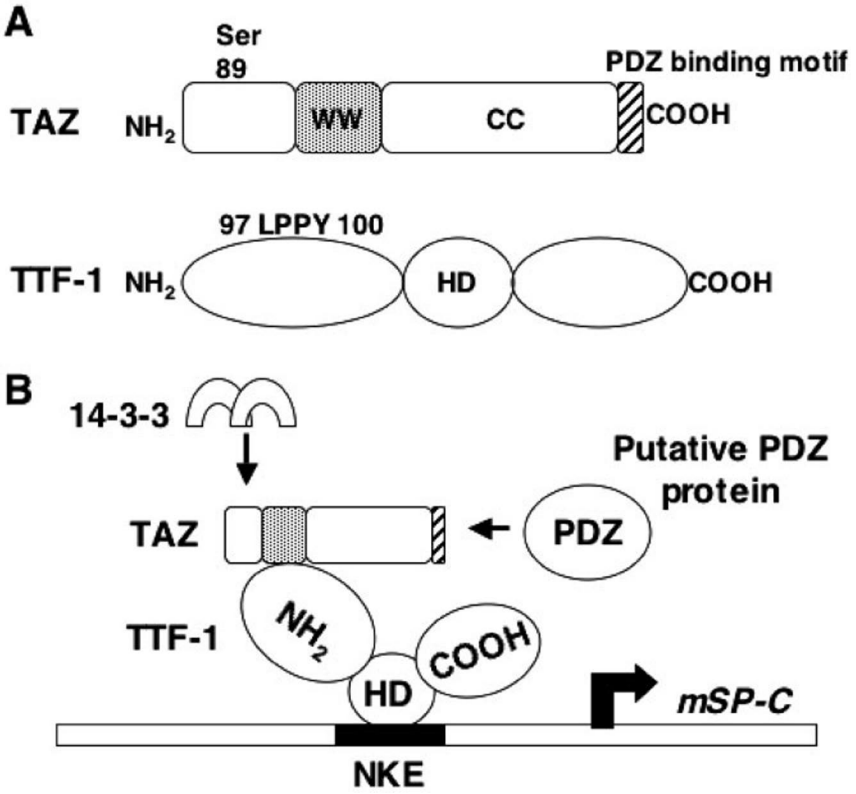

FIG. 6. **Model of TAZ-mediated regulation of TTF-1 transcriptional activity.** The domain structures of TAZ and TTF-1. TAZ contain 1) a WW, (L/P)P*X*Y-binding motif; 2) a CC, coiled-coil domain; 3) a 14-3-3 recognition site at Ser-89 in the NH_2-terminal domain; and 4) a PDZ domain-binding motif in the COOH-terminal domain. TTF-1 contains an (L/P)P*X*Y motif (LPPY) that interacts with the WW domain of TAZ (*A*). The WW domain of TAZ binds to the amino acid sequence LPPY within the NH_2-terminal domain of TTF-1. After phosphorylation of TAZ on Ser-89, TAZ-P binds to 14-3-3-containing proteins in the cytosol, negatively regulating its activity on nuclear transcriptional targets (SP-C). Putative PDZ-containing proteins bind to the COOH terminus of TAZ, promoting nuclear localization, thus positively regulating transcriptional cooperation. *NKE*, NKX binding element; *HD*, the homeodomain of TTF-1; *mSP-C*, mouse SP-C gene (*B*).

scriptional activity on the mouse surfactant protein C promoter (Fig. 6). The enhancement of TTF-1 activity by TAZ was also observed on the activation of an artificial promoter C5-CAT, which contains five consecutive TTF-1-binding DNA sequences.[2] Together with the fact that TAZ is not a DNA-binding protein, functional studies herein suggest that the transcriptional co-activity of TAZ on SP-C expression is achieved through regulation of TTF-1 activity. Mammalian two-hybrid assay and pull-down experiments showed that the functional interaction between TAZ and TTF-1 was likely mediated through direct interactions between the proteins *in vivo*. The NH_2-terminal domain of TTF-1, not the homeodomain nor the COOH-terminal domain, was co-precipitated with TAZ, and contains the four amino acids LPPY, an (L/P)P*X*Y motif, providing a mechanism by which it can bind to WW domain of TAZ. However, both the NH_2-terminal domain and the COOH-terminal domain of TTF-1 are required for full functional interaction with TAZ. RT-PCR, *in situ* hybridization analysis, and immunohistochemistry showed that the temporal-spatial expression of TAZ overlapped with that of TTF-1 and SP-C in respiratory epithelial cells of embryonic and adult mouse lung.

The importance of TTF-1 for normal development of lung and thyroid was well demonstrated in TTF-1-null mice (4, 5). The evidence from the TTF-1-null mice, together with that from studies of TTF-1 overexpression in transgenic mice and patients with TTF-1 haploinsufficiency (18, 17), strongly suggests that lung morphogenesis and function are sensitive to TTF-1 in a dose-dependent manner. TTF-1 action is regulated at both transcriptional and post-translational levels. At post-translational levels, transcriptional activity of TTF-1 is regulated by protein-protein interaction with other nuclear factors (23, 24, 26–29), change of its subcellular localization (41), and phosphorylation (15, 40–43). There have been a number of nuclear proteins interacting with TTF-1 to positively or negatively influence its activity on target genes. Although distinct NH_2-terminal and COOH-terminal domains of TTF-1 were shown to be involved in the transcriptional activation of target genes, the NH_2-terminal domain of TTF-1 has been proposed to serve as a main transcription activation domain (20, 21), and the activity of the COOH-terminal domain varies, depending on target genes and/or cell types (22). The NH_2-terminal domain of TTF-1 exerts transcriptional activity by recruiting various transcription factors and co-factors including Pax8, CBP/p300, SRC-1, and C/EBPα (23, 43, 44). In the present study, TAZ binds to the NH_2-terminal domain of TTF-1, not the homeodomain nor the COOH-terminal domain, but both the NH_2-terminal domain and the COOH-terminal domain are required for the functional interaction with TAZ.

Although TAZ S89A, a mutation on the NH_2-terminal domain of TAZ that lacks the 14-3-3-binding site, further enhanced transcriptional co-activity of TAZ in TTF-1-mediated gene activation, TAZ-4, deletion of the PDZ-binding domain at the COOH terminus of TAZ, inhibited TTF-1 dependent-activity by TAZ. These results were consistent with the previous study in which TAZ S89A increased co-activity of TAZ on the transcriptional activity of a GAL4 DNA-binding domain-PPXY fusion protein (G-PY33) and TAZ-4 eliminated the TAZ-mediated transcriptional co-activation (30). It was therefore suggested that endogenous 14-3-3 and unidentified nuclear PDZ domain proteins regulate transcriptional co-activation function of TAZ through its cytoplasm-nuclear partitioning (30). The present study suggests that the same positive or negative regulatory mechanisms involving 14-3-3 and PDZ domain proteins may modulate TTF-1 mediated expression of surfactant proteins through transcriptional co-activity of TAZ.

Although TTF-1 is expressed both in lung and in thyroid, TTF-1 activates distinct tissue-specific genes in those organs. Although the mechanisms by which the tissue-specific gene is regulated remain unclear, the combinatorial effects of TTF-1 with other nuclear factors whose expression is tissue-specifically regulated have been proposed. Recently, interactions between PAX-8 and TTF-1 were shown to mediate expression of thyroid-specific genes (23). In the present study, we found that TAZ is expressed in the developing and adult lung but was

absent in the thyroid.[3] Therefore, TAZ may play a role in conferring lung-cell specificity of TTF-1 on target genes.

TAZ contains several distinct domains including WW, putative coiled-coil, and PDZ-binding domains, all of which are likely to be involved in protein-protein interactions. The WW domain binds to (L/P)P*X*Y motifs on transcription factors, and a strong intrinsic ability of TAZ to activate transcription was attributed to the region COOH-terminal to the WW domain (30), where a coiled-coil domain may form non-covalent bonds with other transcriptional activators or co-activators. The PDZ domain may mediate localization in the nucleus and the formation of punctate nuclear bodies. The potential roles of the three domains have become evident in a recent study by Cui *et al.* (45) showing that TAZ also acts as a transcriptional co-activator binding to the osteoblast-specific factor Cbfa1. In their study, deletion of any of the three domains significantly reduced, albeit to different extents, the transcriptional co-activity, suggesting that all of the domains of TAZ are necessary for enhancing the transcriptional activation of Cbfa1. The study further suggested that TAZ plays a role in the translocation of Cbfa1 to the nucleus enhanced by other proteins such as the PDZ proteins. Identification of TAZ-interacting proteins will be crucial to study the mechanism of TAZ action, since TTF-1 binds and functions cooperatively with transcription factors such as GATA-6, NFI-B, retinoic acid receptor/retinoid X receptor, C/EBP, and cofactors such as CBP/p300, SRC-1, and TIF-1 in the activation of TTF-1 target genes including *Sftp-a, b, c,* and *ccsp* (24, 26, 27, 43, 46). NFI-B, C/EBP, SRC-1, and TIF2 contain (L/P)P*X*Y motifs, supporting the concept that TAZ may interact with other transcription factors. Whether other factors interact with TAZ in TTF-1-mediated transcription of surfactant protein genes remains to be discerned. Taken together, TAZ binding through its WW domain to the NH_2-terminal domain of TTF-1 may recruit other transcription factors or co-factors to TTF-1 to enhance nuclear localization and activity of TTF-1.

TTF-1 is a critical transcription factor controlling lung morphogenesis and differentiation of respiratory epithelial cells. The present findings demonstrate that TAZ acts as a transcriptional co-activator with TTF-1 in the activation of the surfactant protein C gene. Expression of SP-C is controlled during lung morphogenesis and is regulated for normal surfactant function. Recent studies demonstrated that deletion of SP-C caused severe interstitial lung diseases in mice. Furthermore, mutations in the SP-C gene and abnormal SP-C cause severe interstitial lung diseases in infants, children, and adults. Future studies of TAZ in the context of surfactant protein gene expression, including identification of TAZ-interacting proteins, will give insight to delineating the complex mechanisms that regulate TTF-1 activity in the regulation of surfactant protein C (47–49).

[3] M. Zannini, unpublished observation.

REFERENCES

1. Civitareale, D., Lonigro, R., Sinclair, A. J., and Di Lauro, R. (1989) *EMBO J.* **8,** 2537–2542
2. Guazzi, S., Price, M., De Felice, M., Damante, G., Mattei, M. G., and Di Lauro, R. (1990) *EMBO J.* **9,** 3631–3639
3. Lazzaro, D., Price, M., de Felice, M., and Di Lauro, R. (1991) *Development* **113,** 1093–1104
4. Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C. H., Ward, J. M., and Gonzalez, F. J. (1996) *Genes Dev.* **10,** 60–69
5. Minoo, P., Su, G., Drum, H., Bringas, P., and Kimura, S. (1999) *Dev. Biol.* **209,** 60–71
6. Boshart, M., Klüppel, M., Schmidt, A., Schütz, G., and Luckow, B. (1992) *Gene* (*Amst.*) **110,** 129–130
7. Stahlman, M. T., Gray, M. E., and Whitsett, J. A. (1996) *J. Histochem. Cytochem.* **44,** 673–678
8. Zhou, L., Lim, L., Costa, R. H., and Whitsett, J. A. (1996) *J. Histochem. Cytochem.* **44,** 1183–1193
9. Endo, T., Kaneshige, M., Nakazato, M., Ohmori, M., Harii, N., and Onaya, T. (1997) *Mol. Endocrinol.* **11,** 1747–1755
10. Francis-Lang, H., Price, M., Polycarpou-Schwarz, M., and Di Lauro, R. (1992) *Mol. Cell. Biol.* **12,** 576–588
11. Bruno, M. D., Bohinski, R. J., Huelsman, K. M., Whitsett, J. A., and Korfhagen, T. R. (1995) *J. Biol. Chem.* **270,** 6531–6536
12. Bohinski, R. J., DiLauro, R., and Whitsett, J. A. (1994) *Mol. Cell. Biol.* **14,** 5671–5681
13. Yan, C., Sever, Z., and Whitsett, J. A. (1995) *J. Biol. Chem.* **270,** 24852–24857
14. Kelly, S. E., Bachurski, C. J., Burhans, M. S., and Glasser, S. W. (1996) *J. Biol. Chem.* **271,** 6881–6888
15. Zhang, L., Whitsett, J. A., and Stripp, B. R. (1997) *Biochim. Biophys. Acta* **1350,** 359–367
16. de Felice, M., Silberschmidt, D., DiLauro, R., Xu, Y., Wert, S. E., Weaver, T. E., Bachurski, C. J., Clark, J. C., and Whitsett, J. A. (2003) *J. Biol. Chem.* **278,** 35574–35583
17. Krude, H., Schutz, B., Biebermann, H., von Moers, A., Schnabel, D., Neitzel, H., Tonnies, H., Weise, D., Lafferty, A., Schwarz, S., DeFelice, M., von Deimling, A., van Landeghem, F., DiLauro, R., and Gruters, A. (2002) *J. Clin. Invest.* **109,** 475–480
18. Wert, S. E., Dey, C. R., Blair, P. A., Kimura, S., and Whitsett, J. A. (2002) *Dev. Biol.* **242,** 75–87
19. Damante, G., Fabbro, D., Pellizzari, L., Civitareale, D., Guazzi, S., Polycarpou-Schwartz, M., Cauci, S., Quadrifoglio, F., Formisano, S., and Di Lauro, R. (1994) *Nucleic Acids Res.* **22,** 3075–3083
20. De Felice, M., Damante, G., Zannini, M., Francis-Lang, H., and Di Lauro, R. (1995) *J. Biol. Chem.* **270,** 26649–26656
21. Tell, G., Perrone, L., Fabbro, D., Pellizzari, L., Pucillo, C., De Felice, M., Acquaviva, R., Formisano, S., and Damante, G. (1998) *Biochem. J.* **329,** 395–403
22. Ghaffari, M., Zeng, X., Whitsett, J. A., and Yan, C. (1997) *Biochem. J.* **328,** 757–761
23. Di Palma, T., Nitsch, R., Mascia, A., Nitsch, L., Di Lauro, R., and Zannini, M. (2003) *J. Biol. Chem.* **278,** 3395–3402
24. Liu, C., Glasser, S. W., Wan, H., and Whitsett, J. A. (2002) *J. Biol. Chem.* **277,** 4519–4525
25. Yan, C., Naltner, A., Martin, M., Naltner, M., Fangman, J. M., and Gurel, O. (2002) *J. Biol. Chem.* **277,** 10967–10972
26. Yan, C., Naltner, A., Conkright, J., and Ghaffari, M. (2001) *J. Biol. Chem.* **276,** 21686–21691
27. Bachurski, C. J., Yang, G., Currier, T. A., Gronstajski, R. M., and Hong, D. (2003) *Mol. Cell. Biol.* **23,** 9014–9024
28. Sever-Chroneos, Z., Bachurski, C. J., Yan, C., and Whitsett, J. A. (1999) *Am. J. Physiol.* **277,** L79–L88
29. Yang, Y. S., Yang, M. C., Wang, B., and Weissler, J. C. (2001) *Am. J. Respir. Cell Mol. Biol.* **24,** 30–37
30. Kanai, F., Marignani, P. A., Sarbassova, D., Yagi, R., Hall, R. A., Donowitz, M., Hisaminato, A., Fujiwara, T., Ito, Y., Cantley, L. C., and Yaffe, M. B. (2000) *EMBO J.* **19,** 6778–6791
31. Sudol, M. (1994) *Oncogene* **9,** 2145–2152
32. Bedford, M. T., Sarbassova, D., Xu, J., Leder, P., and Yaffe, M. B. (2000) *J. Biol. Chem.* **275,** 10359–10369
33. Lupas, A., Van Dyke, M., and Stock, J. (1991) *Science* **252,** 1162–1164
34. Rice, W. R., Conkright, J. J., Na, C-L., Ikegami, M., Shannon, J. M., and Weaver, T. E. (2002) *Am. J. Physiol.* **283,** L256–L264
35. Wilkinson, D. G. (1992) *In Situ Hybridization: A Practical Approach*, pp. 75–83, IRL Press, New York
36. Wert, S. E., Glasser, S. W., Korfhagen, T. R., and Whitsett, J. A. (1993) *Dev. Biol.* **156,** 426–443
37. Andersson, S., Davis, D. L., Dahlback, H., Jornvall, H., and Russell, D. W. (1989) *J. Biol. Chem.* **264,** 8222–8229
38. Naltner, A., Ghaffari, M., Whitsett, J. A., and Yan, C. (2000) *J. Biol. Chem.* **275,** 56–62
39. Wikenheiser, K. A., Vorbroker, D. K., Rice, W. R., Clark, J. C., Bachurski, C. J., Oie, H. K., and Whitsett, J. A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11029–11033
40. Kumar, A. S., Gonzales, L. W., and Ballard, P. L. (2000) *Biochim. Biophys. Acta* **1492,** 45–55
41. Yan, C., and Whitsett, J. A. (1997) *J. Biol. Chem.* **272,** 17327–17332
42. Missero, C., Pirro, M. T., and Di Lauro, R. (2000) *Mol. Cell. Biol.* **20,** 2783–2793
43. Yi, M., Tong, G-X., Murry, B., and Mendelson, C. R. (2002) *J. Biol. Chem.* **277,** 2997–3005
44. Cassel, T. N., Berg, T., Suske, T., and Nord, M. (2002) *J. Biol. Chem.* **277,** 36970–36977
45. Cui, C. B., Cooper, L. F., Yang, X., Karsenty, G., and Aukhil, I. (2003) *Mol. Cell. Biol.* **23,** 1004–1013
46. Naltner, A., Wert, S. E., Whitsett, J. A., and Yan, C. (2000) *Am. J. Physiol.* **279,** L1066–L1074
47. Glasser, S. W., Detmer, E. A., Ikegami, M., Na, C. L., Stahlman, M. T., and Whitsett, J. A. (2003) *J. Biol. Chem.* **278,** 14291–14298
48. Amin, R. S., Wert, S. E., Baughman, R. P., Tomashefski, J. F., Jr., Nogee, L. M., Brody, A. S., Hull, W. M., and Whitsett, J. A. (2001) *J. Pediatr.* **139,** 85–92
49. Nogee, L. M., Dunbar, A. E., III, Wert, S. E., Askin, F., Hamvas, A., and Whitsett, J. A. (2001) *N. Engl. J. Med.* **344,** 573–579