GATA-6 and Thyroid Transcription Factor-1 Directly Interact and Regulate Surfactant Protein-C Gene Expression*

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GATA-6, a member of the GATA family of zinc finger domain containing transcription factors, is expressed in endodermally derived tissues including the lung, where GATA-6 influences the transcription of target genes, TTF-1, and surfactant proteins. Whereas GATA-6 did not directly alter expression of sp-C constructs in HeLa cells, GATA-6 synergistically activated sp-C gene transcription when co-expressed with TTF-1, supporting the concept that GATA-6 and TTF-1 might directly interact to influence target gene expression. GST-GATA-6 directly co-precipitated with TTF-1 after in vitro translation and directly interacted with the TTF-1-binding element in the sp-C promoter. Binding of TTF-1 to GATA-6 required the homeodomain of TTF-1, but optimal interactions with GATA-6 required the homeodomain and either carboxyl- or amino-terminal domains of TTF-1. Interactions between TTF-1 and GATA-6 required the amino-terminal and zinc finger domains of GATA-6. Although GATA-4 also interacted with TTF-1 in two-hybrid assays, GATA-4 was not as active as GATA-6 in the activation of the sp-C promoter with TTF-1. Deletion and substitution mutations between GATA-4 and GATA-6 demonstrated that the carboxyl-terminal zinc finger domain of GATA-6 contributed to its synergistic activation of the sp-C promoter with TTF-1. GATA-6 influenced the activity of the sp-C promoter, binding and acting synergistically with TTF-1.

Lung structure and respiratory epithelial cell gene expression are determined by the actions of a number of nuclear transcription factors, including members of homeodomain, Forkhead, and zinc finger families of proteins (1, 2). Members of these families of transcription factors are expressed in the foregut endoderm during lung morphogenesis and in subsets of respiratory epithelial cells later in development, where they influence the expression of genes selectively expressed in the lung, including surfactant proteins SP-A, -B, -C, and Clara cell secretory protein (CCSP)3 (3–9). Thyroid transcription factor-1 (TTF-1), a member of the family of homeodomain-containing transcription factors, plays a critical role in both lung morphogenesis and in respiratory epithelial cell gene expression. TTF-1 is required for the transcription of surfactant proteins and for the formation and differentiation of epithelial cells lining the peripheral respiratory tract (3, 4, 6, 8, 10, 11). TTF-1, also termed Nkx2.1, is a member of the Nkx family of transcription factors that bind an element termed the NKE, containing 5′-TNAAGTG-3′, found in regulatory regions of target genes (12). Multiple NKEs have been identified and their function defined in the surfactant protein and CCSP gene promoters. These elements are required for the activity of the surfactant promoters. TTF-1 directly activates gene expression when expressed in non-respiratory epithelial cells. There is increasing evidence that TTF-1 functions cooperatively with a number of other transcription factors, forming complexes on regulatory regions of target genes. TTF-1 interacts with retinoic acid receptors (RARs) and associated cofactors (13), nuclear factor-1,2 AP1 family members (14), and BR22 (15). Depending on the sites and context of elements within target genes, TTF-1 can act directly or cooperatively with these various factors to regulate gene expression. Whether these interactions are mediated by direct interactions among the transcription factors, or in DNA complexes with other transcription factors, is unclear at present.

GATA-6 is a member of a family of nuclear transcription factors that binds to GATA containing cis-acting elements via highly conserved zinc finger domains. The GATA family includes two distinct groups of proteins as follows: GATA-1, -2, and -3 are involved in hematopoietic cell differentiation and gene expression; and GATA-4, -5, and -6 influence endodermal and mesodernally derived tissues, including lung and heart (16–21). GATA-6 mRNA was detected in the primitive streak, visceral endoderm, gastrointestinal tract, and lung buds in the early mouse embryo (20). Because all GATA family members share the same highly conserved DNA-binding domain and consensus binding element, WGA T-AR, the functional specificity of GATA family members is thought to be mediated by collaboration with other transcription factors or co-factors to activate transcription of target genes (22–26). For example, in cardiac tissues, GATA-4 is co-expressed with Nkx2.5 (Timan) in early cardiac progenitor cells (25), and there is evidence that only GATA-4, but not GATA-6 or GATA-4, directly interacts and synergizes Nkx2.5 activity.

In previous studies (27), GATA-6 was found to bind to elements in the 5′-flanking region of the ttf-1 gene, enhancing ttf-1 gene transcription. The temporal-spatial expression patterns of GATA-6 and TTF-1 overlap in the developing lung and coincide with the sites of expression of surfactant protein, including SP-C (28). Recent studies (29) demonstrated the critical role of surfactant protein-C in surfactant function and pathogenesis of

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3 The abbreviations used are: CCSP, Clara cell secretory protein; TTF-1, thyroid transcription factor-1; RARs, retinoic acid receptors; EMSAs, electrophoretic mobility shift assays; ANOVA, analysis of variance; GST, glutathione S-transferase; NE, nuclear extract; MLE, murine lung epithelial; p.c., postcoitum; HA, hemagglutinin.

C. Bachurski, unpublished observations.
GATA-6 regulates surfactant protein

hereditary interstitial lung disease. Likewise, GATA-6 activated sp-A gene transcription in vitro (5). The present study was therefore undertaken to test whether GATA-6 influences the activity of the surfactant protein-C promoter and whether GATA-6 and TTF-1 might directly interact via protein-protein interactions. The present study demonstrates physical interactions between GATA-6 and TTF-1 and their synergistic effects on the transcription of the mouse sp-C promoter.

EXPERIMENTAL PROCEDURES

Plasmid—The 320 mouse sp-C plasmid was made previously (6). Mouse GATA-6 and GATA-4 are kind gifts from Dr. J. Molkentin, Children’s Hospital, Cincinnati, OH. Rat TTF-1 was a kind gift from Dr. Roberto DiLauro. GATA-6 deletions, TTF-1 deletions, and GATA-6–GATA-4 fusion proteins were generated by PCR as described in Table I and their sequences verified. The cloning sites used on the pMBD and pVP16 vectors (CLONTECH, Palo Alto, CA) are BamHI and XhoI. The cloning sites used on the pcDNA1.1 (Invitrogen) are EcoRV and XhoI. The cloning sites used on the pCruz HA vector (Santa Cruz Biotechnology, Santa Cruz, CA) are EcoRV and BglII.

Cell Culture, Transfection, and Reporter Gene Assays—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. MLE-15 cells were cultured in HITES medium (RPMI 1640 containing 10 mM Hepes, 5 μg/ml insulin, 10 μg/ml transferrin, 3 × 10−6 M sodium selenite, 1 × 10−6 M β-estradiol, and 200 μg/ml L-glutamine) with antibiotics. The cells were seeded at a density of 2 × 105 cells per well in 6-well plates. The reporter construct m320SPC (1 pmol) was co-transfected with 0.2 pmol of either empty vector (pcDNA1.1) or constructs encoding the GATA-6 or TTF-1 trans-activator and 0.5 μg of pCMVβgal by FuGENE6 (Roche Molecular Biochemicals). After 2 days of incubation, lysates were assayed for β-galactosidase and luciferase activity (Promega, Madison, WI). Light units were assayed by luminometry (Monolol 3010, Analytical Luminescence Laboratory, San Diego, CA). Transfection efficiency was normalized with β-galactosidase activity. Data are presented as means ± S.D. Statistical differences assessed by ANOVA.

Electrophoretic Mobility Shift Assays (EMSA)—EMSA were performed essentially as described (2, 7). Briefly, 5–10 μg of nuclear extract (NE) was incubated in binding buffer (20 μM Tris, pH 7.6, 50 mM KCl, 2 mM MgCl2, 40 ng/ml poly(dI-dC), 10% glycerol, 1 mM fresh dithiothreitol, and 0.5 mM fresh phenylmethylsulfonyl fluoride), and when indicated, unlabeled competitor DNA was added for 5 min on ice. Labeled probe (100,000 cpm) was added, and the mixture was incubated for an additional 10 min on ice. The DNA-protein complex was separated from free probes by nondenaturing polyacrylamide gel electrophoresis. Gels were blotted onto Whatman No. 2MM paper, dried, and exposed to x-ray film.

In Situ Hybridization and Immunohistochemistry—In situ hybridization analyses for GATA-6 mRNA were performed on tissues from fetal mice on postcoitum (p.c.) 13.5 and p.c. 16 using 35S-labeled cDNA probes as described previously (5). A 3.1-kilobase pair mouse GATA-6 cDNA template was utilized to generate 35S-labeled probe, which was reduced to an average size of 200 base pairs by alkaline hydrolysis. Immunohistochemical staining for TTF-1 was performed using rabbit anti-rat TTF-1 antiserum kindly provided by Dr. R. DiLauro as described previously (28).

Mammalian Two-hybrid System Assays—The reporter pG5LUC, pVP16/TTF-1 constructs were kind gifts from Dr. C. Yan, Children’s Hospital, Cincinnati, OH. GATA-6 deletions, TTF-1 deletions, and GATA-6–GATA-4 fusion proteins were generated by PCR and subcloned into pVP16 or pMBD vectors between BamHI/XbaI sites (Table I). Transfection and luciferase assays in HeLa cells and MLE-15 cells were performed as described previously (30).

GST-GATA-6 Co-precipitation Assays—To make GST fusion proteins, mouse GATA-6 DNA were synthesized using the primers 5′-CCG GAG GGA TCC TAC CAG ACC TTC GCC GCC-3′ and primer 5′-GGT ACC CTC GAG TCA GGC CAG CTC ACC-3′ by PCR. The PCR product was digested and subcloned into the pGEX4T-1 GST vector (Amersham Biosciences) between the BamHI and XhoI sites. The plasmids were transformed into B21 bacterial strains for protein expression (13). After 3 h of incubation at 37 °C by 1 mM isopropyl-β-D-thiogalactoside, the bacteria were harvested and resuspended in 1× phosphate-buffered saline, followed by sonication and treatment with 1× Triton X-100 overnight. The proteins were purified by incubation with a 50% slurry of glutathione-Sepharose 4B beads (Amersham Biosciences) for 30 min at room temperature and then eluted from beads using glutathione elution buffer. Protein expression was confirmed by Comassie Blue staining and Western blot using GST-GATA-6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Protein concentrations were determined by Lowry assay. For GST co-precipitation assays, TTF-1 protein was labeled with [35S]methionine using PCR3.0/TTF-1 (kind gift from Dr. C. Yan) as template by In Vitro Transcription/Translation kit from Promega (Madison, WI). Glutathione-Sepharose 4B beads were pre-washed in loading buffer containing 1% Triton X-100, 10% glycerol, 0.5 M NaCl, 1 mM EDTA, 50 mM Tris, pH 8.0, and incubated with ~20 μg of purified GST-GATA-6 or GST alone for 1 h at 4 °C. Beads were washed three times with 1 ml of buffer containing 10% glycerol, 25 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, and incubated in vitro translation products at 4 °C for 2 h. The beads were washed three times with the buffer and samples analyzed after SDS-PAGE and autoradiography.

Biotinylated DNA Pull-down Assay—The 22 TTF-1 binding element in the mouse sp-C promoter (~186–167), 5′-TAG GCC AAG GCC CTG GGC GGC TCT-3′, and the element with mutated TTF-1 binding sites, mtC2, 5′-TAG GCC ACT GGC AGT GGC GGC TCT-3′, were synthesized and labeled with biotin. NE with high levels of HA-GATA-6 and TTF-1 were generated from MLE-15 cells transfected with pHA-HA-gata-6 plasmid 48 h before extraction of nuclear protein. Avidin resin (Promega, Madison, WI) was regenerated with 0.1 M NaPO4, pH 7.0, as instructed by the manufacturer and then washed three times with Trit-EDTA buffer containing 50 mM Tris, 2 mM EDTA, and 10 mM NaCl. Biotinylated C2 or mtC2 probe (1 pmol) was incubated with 5 μl of avidin resin in 50 μl of Trit-EDTA buffer for 20 min at 4 °C. The resin was washed three times with Trit-EDTA buffer and twice with 1× binding buffer (20 mM Tris, pH 7.6, 50 mM KCl, 2 mM MgCl2, 40 ng/ml poly(dI-dC), 10% glycerol, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). The avidin resin-biotinylated DNA probe complex was then mixed with NE in 1× binding buffer for 20 min at 4 °C. After incubation, the resin was washed once in 1× binding buffer, and the proteins bound to the probe were eluted with the same probe (5 μl) that was not labeled with biotin in 1× binding buffer. The eluted proteins were subjected to Western blot, and proteins were detected with an HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and an TTF-1 monoclonal antibody generated in this laboratory.

RESULTS

GATA-6 and TTF-1 Synergistically Stimulate the sp-C Promoter—To test whether GATA-6 influenced the expression of lung-specific genes, GATA-6 alone or GATA-6 and TTF-1 were co-transfected with a plasmid construct consisting of the promoter from the murine surfactant protein-C (sp-C) gene and the luciferase reporter gene (pG5LUC). Although GATA-6 did not significantly enhance the activity of the sp-C promoter, GATA-6 and TTF-1 synergistically activated the mouse sp-C promoter in HeLa cells (Fig. 1A). Potential GATA-binding sites were identified on the sp-C promoter at position −152 to −148 and −42 to −39 that bound to murine lung epithelial cell (MLE) nuclear extract and were located near the C2 TTF-1-binding site.
To test whether the pattern of expression of GATA-6 interact with TTF-1 to modulate gene expression, the TTF-1 was required for the stimulatory effects of GATA-6 on cells but strongly influenced TTF-dependent activity. Thus, GATA-6 did not directly activate the sp-C sequence. The GATA-6 and TTF-1 probes were encompassing the GATA motif. The GATA-6 and TTF-1 probes were co-transfected with a luciferase reporter gene containing the sp-C promoter (Fig. 1B). Taken together, GATA-6 did not directly activate the sp-C promoter in HeLa cells but strongly influenced TTF-dependent activity. Thus, TTF-1 was required for the stimulatory effects of GATA-6 on the sp-C promoter, supporting the concept that GATA-6 might interact with TTF-1 to modulate gene expression.

GATA-6 and TTF-1 Are Co-expressed in Respiratory Epithelial Cells—To test whether the pattern of expression of GATA-6 and TTF-1 was consistent with their potential interactions in the regulation of gene expression in developing lung, in situ hybridization and immunohistochemistry were used to identify the sites of expression on postcoitum (p.c.) days 13.5 and 16 of lung development in the mouse. GATA-6 mRNA and TTF-1 proteins were co-expressed in respiratory epithelial cells in the developing lung, suggesting that the two transcription factors might cooperate to regulate gene expression (Fig. 2). Sites of co-expression of TTF-1 and GATA-6 are also consistent with the distribution of SP-C mRNA and protein in the developing lung (28).

GATA-6 and TTF-1 Interact in Both HeLa and MLE-15 Cells—To test whether GATA-6 and TTF-1 might interact in vivo, a mammalian two-hybrid assay was established in HeLa cells and MLE-15 cells, the latter cell line expressing surfactant proteins, GATA-6 and TTF-1 (31). A GATA-6/Gal-4 DNA binding domain fusion protein (pMBD/gata-6) and TTF-1/VP-16 transactivation domain fusion protein (pVP16/tff-1) were co-transfected with a luciferase reporter gene containing four Gal-4-binding sites (pG5Luc) (30). When pMBD/GATA-6 and pVP16/TTF-1 fusion proteins were co-transfected in HeLa cells, luciferase expression was enhanced ~44-fold, demonstrating direct interactions between the gata-6 and tff-1 fusion protein constructs. Reporter gene expression was enhanced 10-fold when the fusion proteins were co-transfected in MLE cells (Fig. 3). The lower activity in the latter cells likely reflects competition between the endogenous GATA-6 and TTF-1 with the fusion proteins, because MLE-15 cells express both transcription proteins.

GATA-6 and TTF-1 Proteins Directly Interact in Vitro—To identify possible direct protein-protein binding between GATA-6 and TTF-1, co-precipitation assays were performed with GST-labeled GATA-6 and TTF-1 produced by in vitro translation. The glutathione-Sepharose 4B beads loaded with GST-GATA-6 retarded the 35S-labeled TTF-1 protein (Fig. 4, lane 2), whereas the beads loaded with GST protein did not retard TTF-1 (Fig. 4, lane 3), confirming the direct interaction between GATA-6 and TTF-1 in vitro.

GATA-6 and TTF-1 Interact at TTF-1-binding Sites in the sp-C Promoter—To test whether GATA-6 interacts with TTF-1 when TTF-1 binds to its binding sites in the mouse sp-C promoter, a biotinylated DNA pull-down assay was performed. The C2 TTF-1-binding element from the sp-C promoter co-precipitated TTF-1 and HA-GATA-6 proteins in vitro. Mutation of the TTF-1-binding sites inhibited the ability of the probe to
bind both TTF-1 and HA-GATA-6 protein, although some residual interaction was observed (Fig. 5). These results suggested that the interaction between GATA-6 and TTF-1 may modify DNA binding activity by TTF-1 and contribute to the synergistic activation by GATA-6 and TTF-1 of the mouse sp-C promoter.

Mapping of GATA-6 Domains Required for the Interaction with TTF-1—To identify the sites required for interactions between GATA-6 and TTF-1, plasmids encoding distinct GATA-6 mutations were subcloned into the pVP16 vector, and interactions between the mutant fusion proteins and TTF-1 in HeLa cells were assessed by two-hybrid assays. After deletion of the zinc finger domain (G6ΔZn) or the amino-terminal domain (G6ΔN), binding to TTF-1 was absent. Likewise, only the zinc finger and amino-terminal domains together (G6ΔC), but not the zinc finger domain alone (G6ΔZn), supported the interaction with TTF-1. Therefore, both the amino-terminal and zinc finger domains of GATA-6 were required for optimal protein interactions with TTF-1, suggesting that the whole amino-terminal domain contributes to conformations of the GATA-6 protein required for its interaction with TTF-1 (data not shown).

Mapping of TTF-1 Domains Required for the Interaction with GATA-6—The amino-terminal (T1-N), homeodomain (T1-HD), and carboxyl-terminal (T1-C) domains of TTF-1 were tested for their interactions with GATA-6 in HeLa cells by two-hybrid assays. Among the three domains, only the homeodomain supported binding to GATA-6. Furthermore, the deletion of amino-terminal domain (T1N) or carboxyl-terminal domain (T1C) of TTF-1 did not block interactions with GATA-6. Therefore, the homeodomain of TTF-1 was required for binding to GATA-6, although optimal binding via the homeodomain required the presence of either the carboxyl-terminal or amino-terminal flanking domains (Fig. 7). The presence of the carboxyl-terminal or amino-terminal flanking domains may contribute to the correct conformation of the TTF-1 homeodomain.

GATA-4 Does Not Strongly Synergize with TTF-1 in the sp-C Promoter—Previous studies have shown that GATA-6 cannot substitute GATA-4 for interaction with Nkx2.5 (23–26). In the present study, we tested whether GATA-4 bound and synergistically enhanced transcription of TTF-1 in the sp-C promoter. In the presence of TTF-1, GATA-4 was not as active as GATA-6 in activation of mouse sp-C promoter, although GATA-4 and TTF-1 interacted in two-hybrid assays (Fig. 8).

The Carboxyl-terminal Zinc Finger of GATA-6 Is Required for Synergy with TTF-1 in the sp-C Promoter—Previous studies demonstrated that the carboxyl-terminal zinc finger of GATA-4 mediated binding with Nkx2.5 (23–26). To determine whether the same domain from GATA-6 mediates the synergy with TTF-1 in the activation of the sp-C promoter, a mutated GATA-6, in which the carboxyl-terminal zinc finger domain was replaced with that from GATA-4 (G646), was generated. The fusion protein was not as active as wild type GATA-6 in synergy with TTF-1 in the mouse sp-C promoter although it still bound to TTF-1, suggesting the carboxyl-terminal zinc finger of GATA-6 contributes to the synergistic activation but is not required for the protein-protein interaction with TTF-1 (Fig. 9). Therefore, the carboxyl-
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**FIG. 6.** Identification of GATA-6 domains required for the interaction with TTF-1. A series of GATA-6 deletion constructs was produced as shown. The GATA-6 deletion mutations were fused with pVP16 vector and co-transfected with pMBD/\textit{ttf}-1 in the mammalian two-hybrid assays to test for the interaction with TTF-1. Both the amino-terminal and the zinc finger domains of GATA-6 are required for the interactions with TTF-1. Values are means ± S.D., \( n = 4 \).

**FIG. 7.** Identification of TTF-1 domains required for the interaction with GATA-6. A series of TTF-1 deletion constructs was produced as shown. The deletion mutations were fused with pVP16 vector and co-transfected with pMBD\textit{gata}-6 in the mammalian two-hybrid assays to test for interaction with GATA-6. The homeodomain of TTF-1 is required for the interactions with GATA-6.

**FIG. 8.** GATA-4 interacts with TTF-1 but is not as active as GATA-6 in synergistic activation of the sp-C promoter. \( A \), the luciferase reporter pG5luc was co-transfected with the pairs of pVP16/\textit{gata}-4 + pMBD/\textit{ttf}-1 into HeLa cells. Individual transfection controls are pVP16 + pMBD/\textit{ttf}-1 and pVP16/\textit{gata}-6 + pMBD/\textit{ttf}-1. Luciferase activity was measured 48 h later. Values are means ± S.D., \( n = 4 \). B, 320map-C was co-transfected with 1 pmol of GATA-6, GATA-4, alone or with TTF-1 into HeLa cells. Cells were harvested, and luciferase and \( \beta \)-galactosidase activities were measured after 48 h. Relative light units were normalized to \( \beta \)-galactosidase activity. GATA-6 was significantly more acute than GATA-4 in synergy with activation of the sp-C promoter with TTF-1, \( *, p < 0.005 \) by ANOVA compared with the group transfected with GATA-6. Values are means ± S.D., \( n = 6 \).

**DISCUSSION**

GATA-6 and TTF-1 synergistically stimulated the activity of the surfactant protein C promoter. Direct interaction of the two transcription factors was observed by co-precipitation and mammalian two-hybrid assays. The binding interaction was dependent upon the homeodomain region of TTF-1 and the amino-terminal and zinc finger domains of GATA-6. Whereas the zinc finger domains of GATA-4 and GATA-6 were highly conserved, the carboxyl-terminal zinc finger domain of GATA-6 conferred more active synergistic activation of the sp-C promoter in the presence of TTF-1. Direct interactions between TTF-1 and GATA-6, and their co-expression during morphogenesis of the lung, support the concept that protein-protein interactions between these members of the distinct transcription proteins may play a role in the expression of target genes in the respiratory epithelium.

Whereas neither GATA-6 nor TTF-1 expression is confined to the lung during development, these transcription factors are co-expressed only in peripheral respiratory epithelial cells of the developing lung, where they cooperatively activated the expression of the sp-C promoter. The sites of co-expression are also consistent with the cell-selective expression of pro-SP-C and other surfactant proteins, including surfactant proteins A and B. TTF-1 is expressed in the forebrain and thyroid and does not overlap with the expression pattern of GATA-6 in these non-respiratory tissues (32). TTF-1 and GATA-6 were also co-expressed in MLE-15 cells, consistent with previous findings demonstrating the requirement of TTF-1 for the expression of surfactant proteins \textit{in vitro} and \textit{in vivo}. In contrast to TTF-1, GATA-4 was not sufficient for activation of sp-C gene expression in HeLa cells; however, the two proteins synergistically activated the sp-C promoter, demonstrating distinct roles of the GATA-6 and TTF-1 elements within the mouse sp-C promoter. Consensus elements for the binding sites for GATA-6 (–152 to –148 and –42 to –39) and TTF-1 (–197 to –158) are present on the mouse sp-C promoter, consistent with the ability of each protein to bind directly at their cis-active elements (6).

The close apposition of the NKE and GATA elements in the sp-C promoter also supports possible direct and indirect interactions between these two factors within the sp-C promoter.

Studies in the cardiac specific gene expression and the expression of atrial natriuretic factor have provided precedents for the interaction between GATA and Nkx family members (22). GATA-4 and Nkx2.5 (tinman) synergistically activated cardiac \( \alpha \)-actin gene expression \textit{in vitro}, and direct interactions
between the two proteins were observed in \textit{in vitro} co-precipitation assays (25). The interactions between Nkx2.5 and GATA-4 were also dependent upon the zinc finger domain in GATA-4 and the homeodomain of the Nkx2.5, findings similar to those presently observed between TTF-1 and GATA-6. These data and others studies (23, 24, 26) support the role of zinc finger domain as a protein-protein interaction domain.

Because the zinc finger of GATA-6 mediated both DNA binding and interaction with TTF-1, the nature of these interactions was assessed by substitution mutations, exchanging the carboxyl-terminal zinc finger of GATA-4 for that of GATA-6 by insertion of G646. Whereas this GATA-6 mutant maintained DNA binding activity to the consensus GATA site, its ability to activate the \textit{sp-C} promoter in a synergistic manner was decreased. The loss of synergy of the GATA-6 (G646) mutant with TTF-1 is likely mediated by impaired interactions between TTF-1 and the carboxyl-terminal zinc finger domain of GATA-6 rather than by changes in DNA binding, because the DNA binding domain is intact. However, both pVPGATA-4 and pVPG646 interacted with TTF-1 in the two-hybrid assay. Thus, the activity of GATA-6 to bind to TTF-1 was not directly correlated with the ability to stimulate synergistically the \textit{sp-C} promoter, suggesting multiple mechanisms by which GATA-6 may influence its activity.

Because TTF-1 is known to interact with a number of other transcription factors with binding sites located in close proximity to the NKEs, TTF-1 and GATA-6 are likely to interact in complex ways at both cis-acting elements with each other and with other protein partners within complexes in the promoters of the target gene. TTF-1 binds and interacts with BR22, RARs, and CBP/P300 in complexes involved in the activation of the \textit{sp-B} gene promoter (15, 30, 33). Likewise, TTF-1 binds and synergistically activates the \textit{sp-C} promoter with nuclear factor-1, binding at sites located –307 to –91, in close proximity to both GATA-6 and TTF-1-binding sites.2 Because GATA-6, TTF-1, NF1, RARs, and other co-factors change developmentally, and may also influence histone acetylation, distinct transcriptional complex may serve to regulate target gene expression during lung development. Furthermore, cis-active elements binding AP-1, NF1, and CREB overlap with the NKE sites in various surfactant protein promoter elements. Interestingly, although GATA-4 interacts with TTF-1, it does not synergistically activate the mouse \textit{sp-C} promoter in HeLa cells as well as GATA-6, suggesting that the binding is not sufficient for synergy between GATA and Nkx family members. Activation by GATA-6 or GATA-4 may be mediated by various mechanisms, including direct binding to GATA elements or by the formation of transcription complexes mediated by direct or indirect interactions between TTF-1 and GATA-6 or among TTF-1, GATA-6, and other transcription factors.

Both GATA-6 and TTF-1 play important but distinct roles in lung morphogenesis and gene expression. TTF-1 is required for the formation of the lung periphery during lung morphogenesis, \textit{tff-1} (–/–) mice have severely hypoplastic lung rudiments, lacking peripheral lung parenchyma at birth (10). Furthermore, TTF-1 is required for expression of surfactant proteins A–C and CCSP in the respiratory epithelium \textit{in vitro} and \textit{in vivo} (6, 8, 34, 35). GATA-6 regulates \textit{tff-1} gene transcription itself, binding and activating the \textit{tff-1} gene promoter (27). GATA-6 is required for endoderm formation \textit{per se}, and targeted deletion of GATA-6 was lethal early during embryogenesis before lung bud formation (36), although recent studies (37) suggested that it may function later in lung morphogenesis. Surprisingly, GATA-6 plays distinct roles in the expression of surfactant protein B, inhibiting TTF-1-dependent activation of the \textit{sp-B} promoter (data not shown), and may therefore play distinct roles in the regulation of various target gene promoters. Whether these distinct effects of GATA-6 on surfactant protein B and C gene transcription contribute to distinct patterns of expression of pro-SP-B and pro-SP-C in the developing lung remain to be discerned.

Thus, TTF-1 and GATA-6 interact in complex ways at cis-active elements of target genes, TTF-1 and GATA-6 bind directly to NKE and GATA elements, located in close proximity.
within the surfactant protein C promoter. GATA-6 and TTF-1 can directly interact through the zinc finger domain of GATA-6 and the homeodomain of TTF-1. Furthermore, TTF-1 forms complexes with other transcription factors, including NF1, RARs, and co-factors, to influence the activity of the surfactant protein C promoter.

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