Nuclear localization domain of thyroid transcription factor-1 in respiratory epithelial cells

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Thyroid transcription factor-1 (TTF-1) is a homeodomain containing transcription factor that binds to and selectively activates the expression of genes in thyroid and pulmonary epithelial cells. TTF-1 plays a critical role in gene expression and in organogenesis of lung and thyroid. In the present work, epitope-tagged TTF-1 proteins were used to identify the regions of the TTF-1 polypeptide that mediate nuclear localization and transcriptional activity in human lung adenocarcinoma cells. A series of TTF-1-flag deletion mutants was generated and transfected into H441 cells to determine amino acid sequences involved in translocation to the nucleus. Transfection of the TTF-1-flag mutants demonstrated that a nuclear localization signal (NLS) sequence, located at the N-terminus of the homeo-

domain, is critical for nuclear targeting. The NLS was essential but not sufficient for translocation of TTF-1 to the nucleus, since deletion of the homeodomain itself also blocked nuclear translocation in the presence of NLS. Deletion of the N-terminal transactivation domain of TTF-1 completely abolished its transcriptional activation on the human surfactant protein-B promoter, and deletion of the C-terminal domain partially reduced its stimulatory activity. Nuclear translocation of TTF-1 depends on both an NLS and the homeodomain of the polypeptide. Both C- and N-terminal regions of TTF-1 are involved in transactivation of surfactant protein B gene expression in pulmonary cells.

INTRODUCTION

Thyroid transcription factor 1 (TTF-1, also termed TTF-1/T-EBP/NKx2.1) is a homeodomain (HD) containing transcription factor controlling both morphogenesis and gene expression in thyroid and lung. TTF-1 protein binds to DNA consensus sequences (the core sequence is CAAG) located with promoter regions of both thyroid- and lung-specific genes, activating gene expression. In the lung, TTF-1 activates the expression of surfactant protein A (SP-A), B (SP-B), C (SP-C) and Clara cell secretory protein genes in epithelial cells [1–5]. Surfactant proteins are essential in maintaining surfactant homeostasis, preventing the lung from collapsing during expiration [6]. In the thyroid, TTF-1 regulates thyroid-specific gene expression, including the thyroglobulin and thyroperoxidase genes [7,8]. Northern blot, *in situ* hybridization and immunohistochemical staining assays demonstrated that TTF-1 is expressed at the onset of lung and thyroid formation [9]. Furthermore genetic ablation of the TTF-1 gene in the mouse by homologous recombination in embryonic stem cells produced offspring with hypoplastic lungs and thyroid agenesis, resulting in death at birth [10]. In addition to its actions in lung and thyroid, TTF-1 is also required for ventral forebrain and pituitary development [10]. TTF-1 is an HD (at positions 160–215) containing transcription factor that is well conserved at both amino acid and DNA levels across various species (> 95 % homology) [11]. Both stimulatory and inhibitory domains have been identified in the TTF-1 polypeptide [12]. TTF-1 is a phosphoprotein and regulated by protein kinase A [13]. TTF-1-DNA binding and consensus sequence recognition are mediated by the HD [14].

Immunohistochemical studies have demonstrated the nuclear localization of TTF-1 in target tissues [9,11]. Short amino acid stretches, termed nuclear localization signals (NLSs), are known to mediate the nuclear import of some proteins [15]. NLSs are highly enriched in basic amino acids and contain both single and

bipartite stretches of basic residues [16,17]. In the present work, TTF-1-flag constructs were used to identify regions of the molecule involved in its transport to the nucleus in human lung adenocarcinoma cells NCI-H441 (H441). In addition, distinct TTF-1 transactivation domains were identified in the N- and C-terminal regions of the TTF-1 polypeptide that are involved in the activation of SP-B gene transcription.

MATERIALS AND METHODS

Cell culture

H441 cells 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 passaged weekly.

Construction of expression vectors

The wild-type (U1-D1) and deletion (U1-D2, U1-D3, U2-D1, U2-D2 and U3-D2) TTF-1-flag expression constructs were made as follows: two primers were synthesized and used for PCR amplification using a rat TTF-1 expression plasmid CMV-TTF-1, kindly provided by Dr. R. Di Lauro (Stazione Zoologica Anton Dohrn, Italy), as a template. In each pair, the upstream primer contains a Kozak sequence and a 21-mer matching the 5'-end of TTF-1 cDNA sequence. The downstream primer contains a 21-mer matching the 3'-end of TTF-1 cDNA sequence and an in-frame flag sequence before two stop codons. Various upstream and downstream primers are as follows. D1 primer: 3'-AACGAAATACCAGCCTGGACCCTGATGTTCTGCTGCTACTGTTCACTATT-5'; D2 primer: 3'-ATGTTCTACTTCGCGGTCCGCTGATGTTCTGCTGCTACTGTTCACTATT-5'; D3 primer: 3'-CGTGGGGCGGCCTTCGCAGCCCTGATGTTCTGCTGCTACTGTTCACTATT-5'; U1 primer: 5'-GCCACCATGTTCGATGAGTCCAAAGCACACGA-

Abbreviations used: TTF-1, thyroid transcription factor-1; NLS, nuclear localization signal; SP-A, surfactant protein A; SP-B, surfactant protein B; SP-C, surfactant protein C; HD, homeodomain; SV40, simian virus 40.

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Figure 1 TITF-1-flag fusion protein

TITF-1-flag fusion protein constructs (wild-type and deletion mutants) were generated for transactivation and translocation studies. HD, homeodomain (black bars); NLS, nuclear localization signal (light shaded bars). The flag sequence at the C-terminus is represented by deep shaded bars. Numbers are amino acid positions.

CT-3'; U2 primer: 5'-GCCACCATGGCACCCCGCCGGAAGCGTCGG-3'; U3 primer: 5'-GCCACCATGCTCTTCTCCAGGCGCAGGT-3'.

The underlined nucleotides represent the flag sequence. The italicized nucleotides represent the Kozak sequence. The PCR products of TITF-1 with the Kozak and flag sequences were directly subcloned into the pCR3 TA cloning vector (Invitrogen) to generate various lengths of pCR3/TITF-1-flag expression constructs. The orientation and DNA sequence of the constructs were confirmed by DNA sequencing using T7 sequenase from USB.

Transfection and luciferase assay

To examine the transactivation activity of TITF-1-flag constructs on the human SP-B promoter luciferase reporter gene, transient transfection assays were performed. Briefly, 2×10^5 H441 cells were seeded per well in six-well plates. Human SP-B promoter (hSP-B-500) reporter construct [1] (3 μ g) and various TITF-1-flag constructs (3 μ g) were transfected into H441 cells by the calcium phosphate precipitation method as described previously [1,18]. In each transfection, 1.6 μ g of CMV- β gal plasmid was included for normalization of transfection efficiency. After 2 days of incubation, cell lysis and luciferase assays were performed using the luciferase assay system (Promega). The light units were assayed by luminometry (monolight 2010; Analytical Luminescence Laboratory, San Diego, CA, U.S.A.). Each experiment was repeated at least three times.

Immunofluorescence

H441 cells were seeded on to Permanox chamber slides (Fisher) at a density ranging from 10^4 to 10^5 per chamber (two chambers per slide). TITF-1-flag plasmids (2 μ g) were transfected into cells using the lipofectin transfection kit from BRL. At 2 days after transfection, the slides were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min, followed by three washes with PBS. Cells on slides were permeabilized with methanol for 2 min and washed four times (5 min each time) with PBS/Triton.

The slides were blocked with 10% goat serum at room temperature for 2 h. The slides were subsequently incubated with 20 μ g/ml anti-flag monoclonal antibody (Kodak) plus 10% goat serum overnight at 4 °C. Next day the slides were washed four times (5 min each time) with PBS/Triton. FITC-conjugated goat anti-mouse IgG antibody (1:100 dilution; Jackson ImmunoResearch Laboratories) in 10% goat serum was added to the slides and incubated for 2 h at room temperature, followed by four washes with PBS/Triton, one wash with 0.2 M phosphate buffer, pH 7.4, and one wash with 0.1 M phosphate buffer, pH 7.4. The slides were visualized with a Nikon Microphot-FXA/video system.

Western-blot assay

H441 cells (6×10^5) were seeded in a 60 mm Petri dish. Next day, 6 μ g of various TITF-1-flag plasmids were transfected into the cells allowing protein expression for 2 days. Cells were harvested and lysed in a cell lysis buffer containing 100 mM NaCl, 50 mM Tris/HCl, 1% Triton 100, 0.1% SDS, 0.5% sodium deoxycholate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin and 5 mM PMSF. Cell lysates were boiled in SDS sample buffer, separated on 10% polyacrylamide gel and transferred to a nitrocellulose membrane. After the membrane incubation with anti-flag monoclonal antibody (1:5000 dilution) at 4 °C overnight, protein bands were visualized with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, U.S.A.).

RESULTS

Construction of TITF-1-flag deletion mutants

Like most transcription factors, TITF-1 is synthesized in the cytoplasm and transported into the nucleus. Since nuclei of all H441 cells were intensely stained as demonstrated by direct immunofluorescence of permeabilized cells using a monoclonal antibody generated against rat TITF-1 (results not shown), a flag sequence (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) was attached to the C-terminus of TITF-1 polypeptide for study of the routing

Figure 2 Immunofluorescence staining of TITF-1-flag fusion proteins

H441 cells were transfected with TITF-1-flag constructs as described in the Materials and methods section and stained with anti-flag monoclonal antibody using fluorescence goat anti-mouse immunoglobulin. Cells were photographed with both phase-contrast and immunofluorescence except those in (F). Arrows point to the immunofluorescent nuclei stained for the TITF-1-flag fusion proteins. (A) U1-D1 construct (wild-type); (B) U1-D2 C-terminal deletion; (C) U2-D1 N-terminal deletion; (D) U2-D2 (homeodomain); (E) U1-D3 construct staining; (F) The picture shown in (E) was photographed by direct immunofluorescence. Original magnification $\times 460$; bar = 22 μm .

of TITF-1 mutant peptides as illustrated in Figure 1. To identify TITF-1 nuclear localization and transactivation domains in lung cells, various regions of TITF-1 cDNA were subcloned into the mammalian cell expression vector PCR3 (Figure 1). A potential NLS was identified at positions 160–166 near the N-terminus of TITF-1 HD and was included in all deletion constructs. To ensure translation efficiency, Kozak consensus sequence was placed in-frame to the N-terminus of full-length and deleted TITF-1 polypeptides.

Table 1 Transcriptional activity of wild-type and mutant TITF-1-flag fusion proteins in H441 cells

H441 cells were co-transfected with SP-B-500-luciferase reporter vector and CMV-TITF-1-flag fusion constructs. Luciferase activity was measured 48 h later. Activity after transfection of empty expression vector (PCR3) was defined as 1. Values represent the mean $\pm$ S.D. ($n = 3$). Activity is measured in light units/absorbance of β -galactosidase.

Plasmids	$10^{-4} \times$ Luciferase activity	Fold stimulation
PCR3	2.1 ± 0.35	1.00
U1-D1	8.7 ± 0.47	4.13
U1-D2	4.5 ± 0.10	2.14
U1-D3	1.8 ± 0.76	0.86
U2-D1	2.3 ± 0.20	1.12
U2-D2	1.7 ± 0.87	0.83

Table 2 Transcriptional activity of wild-type and mutant TITF-1-flag fusion proteins in HeLa cells

Co-transfection and luciferase assay were performed as outlined in Table 1. Values represent the mean $\pm$ S.D. ($n = 3$).

Plasmids	$10^{-3} \times$ Luciferase activity
PCR3	0
U1-D1	1.36 ± 0.15
U1-D2	0.21 ± 0.07
U1-D3	0
U2-D1	0
U2-D2	0

Immunofluorescence localization of TITF-1-flag mutants

Monoclonal antibody against flag was used to detect truncated TITF-1-flag polypeptide expression in H441 cells. As shown in Figure 2, all TITF-1-flag constructs were strongly expressed in the transfected H441 cells as assessed by immunofluorescence staining. Interestingly, all TITF-1-flag constructs were detected in the nuclei except those lacking HD even in the presence of NLS (Figures 2E and 2F), indicating that, in addition to NLS, amino acid sequence in the HD also contributed to the nuclear localization of TITF-1.

Transactivation activity of TITF-1-flag constructs

Table 1 shows the transient transfection study of TITF-1-flag constructs in lung H441 cells. The full-length wild-type TITF-1-flag construct stimulated hSP-B-500 transcriptional activity in H441 cells approx. 4-fold. Stimulation of TITF-1-flag fusion protein was similar to that previously observed in the native rat TITF-1 in this cell line [1], indicating that the flag sequence did not interfere with the transcriptional activity of TITF-1. When amino acid sequence C-terminal to the TITF-1 HD was removed (TITF-1-flag, U1-D2), the polypeptide retained partial activity on the hSP-B promoter. Deletion of amino acid sequence N-terminal to the TITF-1 HD (TITF-1-flag, U2-D1) completely inhibited the activity. When amino acid sequences on both C-terminal and N-terminal sides of the TITF-1 HD were removed (TITF-1-flag, U2-D2), the construct was inactive in the transfection assays. Amino acid sequences N- and C-terminal to the

Figure 3 Protein expression of wild-type and mutant TITF-1–flag in H441 cells

H441 cells were transfected with wild-type or various truncated TITF-1–flag constructs. The empty (PCR3.0) vector is a control. The transfected cell lysates were separated on 10% denaturing polyacrylamide gel with protein standard as molecular-mass markers and transferred to a nitrocellulose membrane. Monoclonal antibody against flag sequence was used for Western blot.

TITF-1 HD therefore play critical but distinct roles in mediating the transcriptional activity of the SP-B promoter construct in H441 cells. Previous studies by Di Lauro and colleagues [14] demonstrated that the HD mediates DNA binding of TITF-1. Deletion of the HD as in the U1-D3 construct completely blocked transactivation of the hSP-B promoter, as expected. Thus the TITF-1 molecule contains two distinct transactivation domains involved in transcriptional activation of the hSP-B-500 promoter construct in H441 cells.

The same studies were also conducted in non-TITF-1-expressing HeLa cells (Table 2). The same results and conclusions were obtained although the hSP-B-500–luciferase reporter gene was expressed at a much lower level than in H441 cells.

Protein expression of TITF-1–flag constructs in H441 cells

To examine the stability of truncated TITF-1–flag polypeptides in cells, Western-blot assay was performed using anti-flag monoclonal antibody. Figure 3 shows that truncated polypeptides

were expressed in H441 cells at levels no lower than the wild-type TITF-1–flag. This indicates that abolition and reduction of transactivation of truncated TITF-1–flag did not result from reduced protein expression.

NLS is required for TITF-1 nuclear localization of H441 cells

A putative NLS sequence (PRRKRRV) was noted at the N-terminus of TITF-1 HD at positions 160–166. This sequence strongly resembles the NLS of simian virus 40 (SV40) large T antigen (Figure 4A). As illustrated in Figure 2, TITF-1 HD, containing this NLS, was sufficient for nuclear localization in H441 cells. To confirm that the putative NLS mediated the nuclear localization of the HD, TITF-1 HD construct lacking the NLS (U3-D2) was made (Figure 4A). Polypeptide produced from the U3-D2 construct was no longer restricted to the nucleus as assessed by immunofluorescence staining using anti-flag monoclonal antibody but was readily detected in the cell cytosol (Figure 4C), demonstrating that the NLS was essential for the nuclear localization of TITF-1.

DISCUSSION

The nuclear localization of endogenous TITF-1 was demonstrated in H441 adenocarcinoma cells by direct immunofluorescence staining with monoclonal antibody. TITF-1–flag constructs were used to identify the domains involved in mediating the activation of SP-B transcription, as well as transport of TITF-1 to the nucleus. An NLS and the HD of TITF-1 were required for efficient transport to the nucleus. Distinct C-terminal and N-terminal domains were identified as being involved in the transcriptional activation of the SP-B promoter and are consistent with previous findings in thyroid cells.

In thyroid cells, two independent domains, located on each side of the TITF-1 HD, contributed to the transactivation of thyroid-specific genes including thyroperoxidase and thyroglobulin [12]. Likewise, in the present study, deletion of either side of the TITF-1 HD abolished or reduced transactivation on the lung-specific hSP-B gene promoter. Inhibition of transcription of the C- and N-terminal mutants was not related to the changes in levels of expression of the mutant proteins in the H441 cells or to its aberrant transport. Immunofluorescence staining identified both TITF-1–flag mutants in the nuclei of transfected H441 cells. As previously reported [12], the HD was also found to be critical for TITF-1 transactivation activity in

Figure 4 Immunofluorescence staining of H441 cells transfected with the U3-D2 TITF-1–flag construct

(A) Illustration of U3-D2 TITF-1–flag construct and TITF-1 NLS. (B) U3-D2-transfected H441 cells. The picture was photographed under phase-contrast microscopy. (C) Same cells as in (B), except photographed under immunofluorescence. In both photomicrographs, the original magnification was $\times 460$; bar = 22 μm .

both thyroid and lung cells. TITF-1 HD binds to *cis*-active sites of target genes in thyroid and lung cell lines as assessed by electrophoretic mobility-shift assay [1,14]. Deletion of HD completely inhibited TITF-1 activity in thyroid cells [12] and, in the present study, did not activate the SP-B promoter in luciferase reporter assays (Tables 1 and 2). In addition, the HD in the absence of the NLS was not transported to the nucleus.

HD is encoded by a highly conserved homeobox DNA sequence that is present in many eukaryotic nuclear proteins. HD-containing proteins are transcription factors with important regulatory roles in organ development and gene expression. In many cases, DNA-binding specificity of these proteins resides exclusively in the HD. Other domains, like the POU domain, leucine zippers, LIM domain and zinc fingers, in some cases are co-operatively interactive with the HD in determining the DNA-binding specificity of the entire protein. HDs appear to bind target sequences as monomers rather than dimers. The three-dimensional structure of the HD demonstrated that HDs contain three α -helices, helix I, II and III. Helices II and III form the helix–turn–helix motif. TITF-1 HD as a DNA-binding domain has been extensively studied. Methylation and ethylation interference experiments showed that the HD alone is sufficient to mediate TITF-1 binding to DNA motifs 5'-CAAG-3' and that Gln-50 in TITF-1 HD is required for DNA-binding specificity [14].

NLSs containing both single and bipartite stretches of basic residues have been described [15,16]. An NLS (PRRKRRV) is found at the N-terminus of TITF-1 HD that resembles the NLS of the SV40 T antigen (PKKKRKV). The TITF-1 HD–flag peptide containing the NLS was restricted to the nucleus (Figure 2), and deletion of the NLS completely inhibited nuclear localization (Figure 4). Recently, several cytosolic factors have been identified that mediate the nuclear import of NLS proteins. These include importin α [19], importin β [20], Ran [21,22], nuclear transport factor 2 (NTF2) [23] and hsp70 [24]. Various mechanisms determining nuclear transport of NLS proteins have been recently reviewed [25]. In the first step, the NLS protein is bound via its NLS by importin α in the cytoplasm. This binding may be facilitated or accelerated by hsp70. In the second step, the complex docks with the cytoplasmic periphery of a nuclear pore complex via importin β . In a third step requiring GTP hydrolysis at Ran, NLS traverses a protein-gated channel and is transported into the nucleus. The NLS protein, importin α , NTF2 and Ran–GDP are released into the nucleus, whereas importin β remains associated with the nuclear pore complex.

Previous studies indicated that NLSs are sufficient to mediate transport of some non-nuclear proteins to the nucleus [15]. The NLS for TITF-1 was not sufficient to transport the N-terminal TITF-1–flag fusion polypeptide into the nucleus, the peptide diffusing into the cytoplasm (Figures 2E and 2F). Addition of the

entire TITF-1 HD to these constructs restored nuclear translocation. Thus both the NLS and HD of TITF-1 appear to be required to mediate translocation of TITF-1 into the nucleus.

The flag sequence has been widely used in many protein factor studies. The flag-fusion protein can be easily separated from endogenous native protein by immunoprecipitation and distinctively detected by Western blot. Addition of the flag sequence did not interfere with TITF-1 transactivation of the SP-B promoter or its nuclear localization.

We thank Dr. Susan Wert for assistance with photomicroscopy and Ms. Ann Maher for manuscript preparation. This work was supported by SCOR grant HL56387 (to J.A.W. and C.Y.) and the American Lung Association (to C.Y.).

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