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Functional inactivation of thyroid transcription factor-1 in PCCl3 thyroid cells

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ABSTRACT

Thyroid transcription factor-1 (TTF-1) is a key regulator of thyroid development and function. In order to identify the genes whose expression depends on TTF-1 transcriptional activity within the thyrocyte we analyzed the consequence of the functional inactivation of this factor in PCCl3 cells. The expression of a fusion protein composed of the DNA binding domain of TTF-1 and of the strong repressive domain of the engrailed protein resulted in a dramatic loss of epithelial cell morphology and in proliferation arrest. These changes were reversed when the inhibition of endogenous TTF-1 was relieved. No change was observed when a similar fusion protein containing point mutations abolishing DNA binding activity was produced in the cells. Besides the expected down-regulation of expression of the main genes linked to the differentiated thyroid function, we observed a decreased expression of the transcription factors Hhex, Pax 8 and TTF-2 and of E-cadherin. By contrast, both ThOX-1 and DUOXA-1 genes were up-regulated, as well as the ones encoding vimentin and several proteins involved in cell cycle arrest. Our data thus extend the known roles of TTF-1 in thyroid development and in the expression of differentiated function in the adult organ to the control of epithelial morphology and of cell division in mature thyrocytes.

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1. Introduction

Thyroid transcription factor-1 (TTF-1, also known as Nkx-2.1 or T/ebp or Titf1), the product of the TITF-1 gene, is a homeodomain-containing transactivator playing pivotal roles both in the development of the thyroid (review by De Felice and Di Lauro, 2004) and in the transcriptional control of genes involved in the differentiated function of thyrocytes (review by Damante et al., 2001). Additionally, TTF-1 also plays key roles in the development and function of the lung and in the developing forebrain (De Felice and Di Lauro, 2004). Heterozygous mutations in the human TITF-1 gene are associated with mixed pathologies including congenital hypothyroidism, respiratory distress and neurological disorders (review by Montanelli and Tonacchera, 2010).

Inactivation of the TITF-1 gene in the mouse resulted in the absence of thyroid development which precluded the analysis of its role in the mature organ (Kimura et al., 1996). Only partial conditional inactivation of the gene was later reported in the mouse thyroid, leading to a mitigated phenotype of disorganized and hypofunctional tissue (Kusakabe et al., 2006). The main lesson from the former study is the requirement of TTF-1 for the survival and proliferation of the primitive thyroid cells. The second study indicated that within the developed organ all cells must contain adequate amounts of this factor in order to maintain a normally functioning tissue. Very recently, mice harboring TTF-1 alleles lacking the sequences encoding either the N-terminal or the C-terminal transactivation domain of the protein were generated (Silberschmidt et al., 2011). Animals homozygous for the deletion did not develop a thyroid, confirming the requirement for the transcription-activating function of TTF-1 in this process.

The main known function of TTF-1 in the mature thyrocyte is to activate the transcription of the so-called thyroid-specific genes, the prominent ones being those encoding thyroglobulin (Tg), thyroperoxidase (TPO) and the sodium-iodide symporter (NIS) (Damante et al., 2001). This activity appears to involve a synergy with the transcription factor Pax 8, another protein playing a dominant role in the control of differentiation of the thyrocyte (Di Palma et al., 2003). Contradictory reports exist regarding a possible role for TTF-1 in the control of thyroid cell proliferation as on one side low levels of TTF-1 were associated with goiter formation in man (Acebron et al.,1995) and thyrotropin (TSH) stimulation was reported to down-regulate TTF-1 expression in FRTL-5 thyroid cells (Saito et al., 1997), whereas on the other side the addition of TTF-1 antisense oligonucleotides was reported to counteract the mitogenic effect of TSH in the same cell system (Rossi et al., 1995). More recently, a study conducted on TTF-1 thyroid-conditional hypomorphic mice concluded that this factor "may

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function to control the proliferation of thyroid follicular cells following damage by a genotoxic carcinogen" (Hoshi et al., 2009).

In order to identify the whole set of genes controlled by TTF-1 in the mature thyrocyte we decided to express in the differentiated rat thyroid cell line PCCl3 a TTF-1 antagonist that was developed previously in our lab (Christophe-Hobertus and Christophe, 2007). This antagonist consists in a fusion protein composed of the DNA-binding TTF-1 homeodomain and the strong repressive domain of the drosophila engrailed factor. By competing with the endogenous TTF-1 for binding to the target promoters, the produced antagonist was expected to decrease substantially the expression of the genes that depend on transactivation by TTF-1.

2. Materials and methods

2.1. DNA constructions

Oligonucleotides used for PCR or DNA sequencing were purchased from Eurogentec (Seraing, Belgium). All PCR-amplified sequences were verified by DNA sequencing in order to exclude the presence of sequence changes introduced during the PCR step.

The construction and functional validation of the TTF-1 homeodomain-engrailed repressive domain fusion protein expression plasmids pcDNA3-Engr-HD and pcDNA3-Engr-HDm as well as the reporter construct harboring the Tg promoter have been described previously (Christophe-Hobertus and Christophe, 2007). The sequences encoding Engr-HD and Engr-HDm were subcloned into the bicistronic vector pEFIN4 (a generous gift of Dr. S. Costagliola, Université Libre de Bruxelles, IRIBHM, Brussels, Belgium) after PCR amplification using the following primer pair:forward primer: 5′-GCGAATTCGCCATGGCCCTGGAGGATCGCTG-3′ reverse primer: 5′-GCTCTAGACTACTGCGCCGCCTTGTCC-3′ that added EcoRI and XbaI restriction sites at the 5′ and 3′ borders of the amplicon respectively. The same coding sequences were subcloned between the PvuII and NheI restriction sites in the vector pTRE2hyg (Clontech) after excision from the pEFIN4 constructs with EcoRV and XbaI. The expression plasmid encoding the tetracycline-controlled transactivator (tTA) was obtained by excising the tTA-encoding sequence from plasmid pTR-Tet-Off-EGFP (kindly given by Dr. A. Brandenburger, CMMI, Gosselies, Belgium) using EcoRI and BamHI and inserting it within the corresponding restriction sites in the bicistronic vector pEFIN4.

2.2. Cell culture and DNA transfections

PCCl3 cells were cultured as described by Fusco et al. (1987). Clones stably transformed with a pEFIN4 expression construct were selected in culture medium supplemented with 400 μg/mL of Geneticin (Gibco) and maintained afterward in the presence of 200 μg/mL of Geneticin. For the isolation of tTA-expressing clones transformed with the pTRE2hyg construct driving the conditional expression of Engr-HD or Engr-HDm, cells were transfected in culture medium supplemented with 200 μg/mL of Geneticin and 2 μg/mL of doxycycline (Sigma–Aldrich) and then selected in the presence of 200 μg/mL of both Geneticin and hygromycin (HygroGold, InvivoGen) and 2 μg/mL of doxycycline. TPC-1 and 8505-C cells were cultured as described by van Staveren et al. (2007). COS-7 and Hela cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2.5 μg/mL amphotericin-B, 100 IU/mL penicillin and 100 μg/mL streptomycin, at 37 °C in a water-saturated 95% air/5% CO_2 atmosphere. Transfections or co-transfections were performed as described before (Christophe-Hobertus and Christophe, 2007). The plasmid pRLSV40 (Promega) containing the renilla luciferase gene under the control of the SV40 enhancer-promoter was used in transient transfection experiments as an internal control for normalization in regard to transfection efficiency. The pTRE2hyg-Luc construct (Clontech) was used to select the tTA-expressing PCCl3 clone displaying the best response to doxycycline withdrawal.

2.3. Luciferase assays

Firefly and renilla luciferase activities were measured between 24 and 48 h after transfection using the Dual-Luciferase® Reporter Assay System as recommended by the manufacturer (Promega Corp.) and a TD-20/20 luminometer (Turner Designs, Sunnyvale, California, USA). All transfections were performed in duplicate within each individual experiment. For each reporter construct, the mean ratio (from the duplicates) of firefly luciferase activity to renilla luciferase activity was used as normalized luciferase activity. When data are presented as the ratio of normalized luciferase activity in cells expressing Engr-HD to the normalized luciferase activity in cells expressing Engr-HDm, the Engr-HD/Engr-HDm ratio of the reference construct pGL3control (Promega), harboring the SV40 enhancer-promoter, was set to 1 in each individual experiment, and all the other values were normalized accordingly. Statistical analysis of the data was performed using the two-tailed Mann-Whithney U test available on the Elementary Statistics for Biologist web pages by Leon Avery (http://elegans.swmed.edu/~leon/stats/utest.html).

2.4. Microscopic examination and cell staining

Microscopic examination was performed on an Eclipse TE300 inverted microscope (Nikon) and pictures were recorded using a 3CCD color video camera XC-003P (Sony) at a magnification of 100. Cells in dishes were stained by incubation in 0.5 mg/mL methylene blue solution for 1 h at room temperature followed by two rapid washes with distilled water. Pictures were taken immediately after the last wash using a Canon PowerShot G5 digital camera.

2.5. Western blot analysis

Western blots were performed as described before (Christophe-Hobertus and Christophe, 1999) using the anti-engrailed d300 antibody from Santa-Cruz at a dilution of 1/1000.

2.6. Micro-array experiments and data analysis

Total RNA was obtained from cells at 50% confluency maintained in the presence or absence of doxycycline for one week using the RNeasy mini kit from Invitrogen. Double-stranded cDNA was synthesized from 1 μg of total RNA, followed by production of antisense RNA containing the modified nucleotide 5-(3-aminoallyl)-UTP using the Amino Allyl MessageAmp™ II aRNA Amplification kit (Ambion, Texas, USA). After labeling with Cy3 or Cy5 (GE Healthcare Bio-Sciences, New Jersey, USA), test/control sample pairs were hybridized onto Rat MI ReadyArray (Microarrays Inc., Alabama USA). The oligonucleotides set consists of 34,717 70-mer transcript probes representing genes and alternative splice products from the Operon Biotechnologies' Array Ready Oligo Set version 3.0 for Rattus norvegicus. Hybridizations were replicated with dye swap. Slides were scanned using a Molecular Devices 4000B laser scanner and expression levels were quantified using GenePix Pro 6.1 image analysis software (Axon Instruments, CA, USA). Image acquisitions were performed with automatic photomultiplier gains (PMT) adjustment. Artifact-associated spots were eliminated by both visual and software-guided flags, as well as spots with a signal/background fluorescence ratio less than 2. The fluorescence values were imported into Acuity 4.0 software

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