Function of the Homeo and Paired Domain Proteins TTF-1 and Pax-8 in Thyroid Cell Proliferation*

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The thyroid transcription factors TTF-1 and Pax-8 are homeobox- and paired box-containing genes, respectively, that are responsible for thyroid-specific gene expression, thyroid development, and thyroid cell differentiation. However, it is not clear if such factors play a role in thyroid cell proliferation. The antisense oligonucleotide strategy was used in order to clarify this point. Treatment of quiescent FRTL-5 thyroid cells with TTF-1 or Pax-8 antisense oligonucleotides caused a significant reduction in thyroid-stimulating hormone and insulin-like growth factor-I-stimulated cell proliferation, measured by DNA synthesis and cell counting. The same results were obtained with forskolin indicating that the TTF-1 or Pax-8 role in mediating the thyroid-stimulating hormone growth effect occurred via the cAMP pathway. The effect was higher with TTF-1 as the blockage by this factor caused a 65% decrease in cell proliferation compared to the control. Pax-8 blocking led only to a 30% decrease. The blocking of both thyroid transcription factors together did not result in an additive effect. These data provide direct evidence that both homeo and paired box gene expression is essential for FRTL-5 thyroid cell proliferation, with each one possibly playing a different regulatory role.

Homeo- and paired domain-containing proteins are transcriptional regulators that participate in cell growth and differentiation processes (1, 2). The thyroid-specific transcription factors TTF-1 and Pax-8 are homeo and paired box genes, respectively, that are responsible for thyroglobulin and thyroid peroxidase gene expression (3–6). In addition, TTF-1 binds to the TSH receptor (TSH-R) gene promoter (7, 8). Both transcription factors are responsible for thyroid development, demonstrated by the fact that their expression precedes the onset of their target genes thyroglobulin, thyroid peroxidase, and TSH-R by 5 days (9, 10). Clear evidence for the major role of TTF-1 and Pax-8 in thyroid cell differentiation is that transformed FRTL-5 thyroid cells, where TTF-1 and/or Pax-8 are not expressed (11), loose their thyroid phenotype. It has also been reported that undifferentiated thyroid carcinomas do not express either TTF-1 or Pax-8 (12). However, it has not yet been demonstrated the role of both transcription factors in thyroid cell growth.

The transition from quiescent to proliferating FRTL-5 thyroid cells requires the action of both TSH and IGF-I (13–16). A significant amount of evidence suggests that TSH and IGF-I, could in some way, regulate TTF-1 and Pax-8 activity since mutations in the TTF-1/Pax-8 binding sites of thyroglobulin and thyroid peroxidase promoters reduce their response to TSH and IGF-I (17, 18). The aim of the present work was to study whether TTF-1 and Pax-8 play a role in thyroid cell growth in response to TSH and IGF-I. We used antisense oligonucleotides to inhibit TTF-1 or Pax-8 expression in FRTL-5 thyroid cells. This treatment led to a marked decrease in the rate of DNA synthesis and cell number in FRTL-5 cells in response to TSH and/or IGF-I. The inhibitory effect was higher when TTF-1 was blocked than that observed for Pax-8 blockage. Blocking both transcription factors simultaneously did not result in an additive effect. These data provide direct evidence that both TTF-1 and Pax-8 gene expression is required not only for the establishment and maintenance of the differentiated phenotype, but also is essential for thyroid cell proliferation.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture medium, bovine TSH, bovine insulin, and TPA were purchased from Sigma. Rat IGF-I was from Amgen Biological (Thousand Oaks, CA) and forskolin was from Calbiochem. Donor calf serum was from Life Technologies, Inc., and Nytran and nitrocellulose filters from Schleicher & Schuell. The ECL kit, streptavidin-horseradish peroxidase conjugate, pre-stained protein marker, [3H]thymidine, (γ)32P]ATP and [α-32P]dCTP were from Amersham Corp.

Cell Culture—The FRTL-5 cells (ATCC CRL 8305; American Type Culture Collection, Rockville, MD) used in this study are a continuous strain of functioning epithelial cells derived from normal Fisher rat thyroid. The cells were cultured as described elsewhere (19) in Coon's modified Ham's F-12 medium supplemented with 15% calf serum and a six-hormone mixture including 0.5 milliunit/ml TSH and 10 μg/ml insulin (6H medium). Cells were used, unless otherwise noted, when they approached confluence. Quiescent FRTL-5 cells were obtained after deprivation for 4 days of TSH and insulin by maintaining in 0.2% calf serum (4H medium).

Oligonucleotides—A 24-mer antisense oligodeoxynucleotide complementary to a sequence starting at the ATG initiation codon of rat TTF-1 (5'-GGT GTG CTT TGG ACG ACT CAT CGA CAT-3') or rat Pax-8 (5'-GGA TCT GAT CGA GTT GTG AGG CAT-3') and a 24-mer sense oligodeoxynucleotide corresponding to the same sequences (5'-ATG TCG AGT AGT CAA CAG ACG-3') and (5'-ATG CCT CAC AAC TCG ATC AGA TCC-3'), respectively, were synthesized with an Applied Biosystems 391 DNA synthesizer and purified on acrylamide gels.

Northern Blot Analysis—Total RNA was isolated by the guanidine-thiocyanate-phenol procedure (20) from FRTL-5 cells after different treatments. Samples of total RNA were electrophoresed in 1% agarose gels containing 2.2 μm formaldehyde. RNA was blotted onto nylon filters as suggested by the manufacturer. Methylene blue staining of the blots revealed the integrity of the RNA and the presence of equal amounts in each lane. Hybridization and washing (21) were carried out with specific probes for TTF-1 (4), Pax-8 (6), and c-fos (22) labeled by random oligo priming.

Western Blotting—Nuclear extracts were prepared (23) from FRTL-5 cells maintained under the different study conditions. Protein concentration was determined with the Bio-Rad protein assay kit with bovine...
serum albumin as the standard. Nuclear proteins (20 μg of each sample) were separated by 8% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes using a Bio-Rad transfer apparatus (10 mA/gel, overnight) with 20% methanol, 25 mM Tris base, and 192 mM glycine as the transfer buffer. Membranes were blocked for 1-2 h at room temperature in TBS-T buffer (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween-20, pH 7.5) containing 10% bovine serum albumin. Membranes were incubated at room temperature for 1 h in TBS-T containing 10% bovine serum albumin and α-TTF-1 antibody (1 μg/ml) and then washed three times, 10 min each, with 10 ml of TBS-T buffer with 10% bovine serum albumin. Membranes were then incubated for 60 min in TBS-T buffer plus 10% bovine serum albumin with a 1:3,000 dilution of α-TTF-1 antibody or a 1:5,000 dilution of streptavidin-horseradish peroxidase conjugate as appropriate followed by two 30-min washes with TBS-T buffer. Immunoreactive bands were visualized using an ECL kit as described by the manufacturer.

Electrophoretic Mobility Shift Assays—Nuclear proteins (5 μg) from FRTL-5 prepared as above were incubated with 50 pg of 32P-labeled double-stranded DNA oligonucleotide P (5′-CTA AGC TGG AGT GGG CAT CAG AGC ATG GAG TC-3′) the site at which Pax-8 binds to thyroperoxidase promoter. The binding reactions were performed as described elsewhere (17) except that the salt concentration was reduced to 75 mM KCl, optimal binding conditions for Pax-8 (5, 6). In competition experiments, the unlabeled competitor was added in a 100-fold excess and was always preincubated with the extract for 10 min on ice prior to adding the labeled probe. The resulting DNA-protein complexes were separated from free DNA on a 5% polyacrylamide gel (17).

Cell Proliferation—Quiescent FRTL-5 cells in 24 well plates were incubated for 3 days with sense or antisense oligonucleotides at a final concentration of 5 μM. To measure DNA synthesis, 1 μCi/well of [3H]thymidine was added, and half of the groups were stimulated with a 1:3,000 dilution of α-TTF-1 antibody or a 1:5,000 dilution of streptavidin-horseradish peroxidase conjugate as appropriate followed by two 30-min washes with TBS-T buffer. Immunoreactive bands were visualized using an ECL kit as described by the manufacturer.

RESULTS

Treatment with TTF-1 or Pax-8 Antisense Oligonucleotides Decreases mRNA and Protein Levels of Both Genes in FRTL-5 Cells—In order to demonstrate that the thyroid-specific transcription factors TTF-1 and Pax-8 play a role in thyroid cell proliferation, we used the antisense oligonucleotide approach to FRTL-5 cells, whose transition from quiescence to proliferation requires the action of TSH and IGF-I. Due to the relative stability of TTF-1 and Pax-8 proteins,2 we found it necessary to maintain the cells in the presence of antisense or sense oligonucleotides, respectively. Cells were then harvested, and radioactivity was measured using standard scintillation counting. The same experimental approach was followed in the experiments in which the cells were counted. In this case no [3H]thymidine was added, and the cells were harvested by trypsinization.

Fig. 1. Effect of TTF-1 and Pax-8 antisense oligonucleotides on TTF-1 and Pax-8 mRNA levels. Total RNA from FRTL-5 thyroid cells cultured in control 6H medium or treated 4 days with 5 μM of TTF-1 (A) or Pax-8 (B) sense or antisense oligonucleotides was isolated. Northern blots of total RNA (20 μg) were hybridized with TTF-1 or Pax-8 cDNA probes (top panel). The specificity of the reduction in mRNA levels after each treatment is demonstrated by hybridization with the probe of the alternative transcription factor (middle panel). The sizes of TTF-1 and Pax-8 mRNAs are indicated. The bottom panel is the result of methylene blue staining of the membranes after transfer.

After staining with methylene blue is shown in the bottom panel.

Since the half-life of TTF-1 and Pax-8 mRNAs is short compared to the long half-life of the protein we decided to study whether the antisense treatment also reduced the protein levels. For this purpose we detected the presence of TTF-1 and Pax-8 proteins by Western blotting and electrophoretic mobility shift assay, respectively. Nuclear proteins (20 μg) extracted from confluent FRTL-5 cells or from the same cells treated for 4 days with sense or antisense TTF-1 oligonucleotide were separated in a 8% SDS-polyacrylamide gel electrophoresis, together with a prestained protein marker. Nuclear extracts from RAT-1 fibroblasts were used as a negative control. After protein transfer to nitrocellulose, the Western blot was probed with anti-TTF-1 antibodies. Immunoreactive bands were visualized using the ECL kit. As shown in Fig. 2A, a clear and specific decrease is detected in TTF-1 protein in antisense-treated cells (lane 3) in comparison to cells treated with a control TTF-1 sense oligonucleotide (lane 1). TTF-1 protein, as expected, was not present in RAT-1 fibroblasts (lane 2).

Nuclear extracts (5 μg) from FRTL-5 cells, previously incubated with sense or antisense Pax-8 oligonucleotides, were tested for their ability to bind a 32-base pair oligonucleotide derived from the −30 to −61 region within the thyroperoxidase promoter which has been shown to recognize the transcription factor Pax-8 (5, 6). A retarded band was detected in the gel shift assay when nuclear extracts from both control or sense treated FRTL-5 cells were used (Fig. 2B, lanes 2 and 3). The absence of Pax-8 was demonstrated when nuclear extracts from cells treated with antisense oligonucleotides were used, since no retarded band corresponding to Pax-8 DNA complex was observed (lane 4). The specificity of the complex was demonstrated by competition experiments. The complex was competed by a 100-fold excess of unlabelled oligonucleotide P (lanes 5–7). TTF-1 and Pax-8 Antisense Oligonucleotides Inhibit TSH- and IGF-I-stimulated Thyroid Cell Proliferation—In order to study whether TTF-1 and Pax-8 play a role in FRTL-5 cell proliferation in response to TSH and IGF-I, the following experiments were carried out. Quiescent FRTL-5 cells were incubated with 5 μM TTF-1 and/or Pax-8 sense or antisense oligonucleotides in 24-well plates for 3 days. For the DNA synthesis

2 D. L. Rossi, A. Acebrón, and P. Santisteban, unpublished observation.
Thyroid Cell Growth and Thyroid Transcription Factors

**DISCUSSION**

Different evidence suggests that the thyroid-specific transcription factors TTF-1 and Pax-8, homeo and paired box genes, respectively, are necessary for thyroid phenotype determination, thyroid development (9, 10), as well as for differentiation (11, 12). Since this kind of genes are also involved in cell proliferation (1–2), the aim of this work was to demonstrate that both TTF-1 and Pax-8 are necessary in processes of thyroid cell growth. We have used an effective and simple antisense approach for the specific depletion of TTF-1 and Pax-8 from FRTL-5 cells. The cells treated with sense or antisense oligonucleotides were viable and exhibited apparently normal cellular phenotype. Sense oligonucleotides or scrambled sequences were used as non-blocking controls. The oligonucleotides used in this work were changed every 24 h and appeared to be effective. The controls used for measuring the antisense effect were those recommended previously (24). The target RNAs and proteins levels after antisense oligonucleotide treatment were clearly reduced compared to that of control sense treatment. We consider that the approach used up to this point was appropriate to demonstrate our aim.

Thyroid follicular cells are regulated by a variety of growth-stimulating factors, including TSH and IGF-I. Both growth factors stimulate proliferation and differentiation (13–16, 25, 26). It is well accepted that TSH works through the cAMP pathway in regulating thyroid growth (27–29). Experiments measuring [3H]thymidine incorporation in 24-well plates with 5 μM of TTF-1 (A and C) or Pax-8 (B and D) sense or antisense oligonucleotides for 4 days. Then half of the groups were stimulated with different ligands for another 24 h. A and B, cells were pulse-labeled with 1 μCi of [3H]thymidine as described under “Experimental Procedures,” and DNA synthesis was determined as thymidine incorporation (C and D). Cell counts were performed after trypsinization in the absence of [3H]thymidine as described above. Data are average values ± S.D. of three independent experiments performed in triplicate. □, sense; ■, antisense.

**Fig. 2. Effect of TTF-1 and Pax-8 antisense oligonucleotides on TTF-1 and Pax-8 protein levels.** Nuclear extracts from the different treatments were isolated. A, Western blot analysis with nuclear proteins (20 μg) from FRTL-5 cells treated with TTF-1 sense or antisense oligonucleotides and from RAT-1 fibroblasts were probed with a-TTF-1 antibodies. The size of the TTF-1 protein is shown. B, electrophoretic mobility shift assay from nuclear extracts (4 μg) of control, Pax-8 sense, or antisense treated FRTL-5 cells incubated with the labeled oligonucleotide P (Pax-8-binding site). The specificity of the retarded complex was established by competition with a 100-fold excess of unlabeled oligonucleotide P. The migration of the Pax-8-DNA complex is shown.

**Fig. 3. Effect of TTF-1 and Pax-8 antisense oligonucleotides on FRTL-5 cells proliferation.** Quiescent FRTL-5 cells were incubated in 24-well plates with 5 μM of TTF-1 (A and C) or Pax-8 (B and D) sense or antisense oligonucleotides for 4 days. Then half of the groups were stimulated with different ligands for another 24 h. A and B, cells were pulse-labeled with 1 μCi of [3H]thymidine as described under “Experimental Procedures,” and DNA synthesis was determined as thymidine incorporation (C and D). Cell counts were performed after trypsinization in the absence of [3H]thymidine as described above. Data are average values ± S.D. of three independent experiments performed in triplicate. □, sense; ■, antisense.
thyroid phenotype. When the cells are not totally differentiated, by blocking TTF-1 and Pax-8, they do not respond to growth factors such as TSH and IGF-I. The other possibility, more complex but to us a very interesting explanation, is that both factors play a functional role in the regulation of thyroid cell cycle. This would result in TTF-1 or Pax-8 mRNA being regulated by TSH and IGF-I. Although other authors (8) have reported evidence that TTF-1 mRNA is down-regulated by TSH, in our conditions its mRNA is not regulated either by TSH, forskolin (data not shown) or by IGF-I (17). However, both growth factors could regulate TTF-1 by some other mechanism. One of the possible posttranslational regulation mechanisms is protein phosphorylation. TTF-1 has been shown to be a phosphoprotein (33). Thus, our hypothesis is that at least for this transcription factor, TSH and IGF-I could regulate its phosphorylation state through the pathways used by such factors to induce thyroid cell growth.

The transition from quiescence to proliferation requires the presence of mitogenic stimulation. It is well accepted that different kinases are activated in many cell types in response to growth factors during the G0 to G1 transition in the cell cycle (34, 35). These kinases could phosphorylate either TTF-1 or a cell cycle kinase, such as cdc2, that will be involved in TTF-1 activation. Phosphorylated TTF-1 could regulate one still unknown protein of the cell cycle. We can postulate the same theory in the case of Pax-8. It has been suggested (2) that Pax genes might be involved in growth regulation because cell cycle proteins, such as cdc2, whose steady state levels are growth regulated would be good candidates for common targets of developmental control (36, 37).

To determine if the mechanism by which blocking TTF-1 mRNA interferes with DNA synthesis could implicate the regulation of genes involved in the proliferation, we analyzed the mRNA levels of c-fos. The results obtained (not shown) indicated that c-fos, a gene involved in FRTL-5 cell proliferation, remained unaffected in TTF-1 antisense-treated cells after TSH or IGF-I stimulation. At present, we can only speculate about the precise function of both thyroid transcription factors in the cell cycle control but it seems reasonable that it might regulate another set of genes expressed later in the growth response. The demonstration of this hypothesis will require a more careful study of possible targets of TTF-1 or Pax-8 action.

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