The Functional Interaction Between the Paired-Domain Transcription Factor Pax8 and Smad3 is Involved in the TGF-β Repression of the Sodium Iodide Symporter Gene

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Running title: TGF-β/Smad3 represses NIS gene expression

Key words: NIS, TGF-β, Pax8, Smads, thyroid

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ABSTRACT
Transforming growth factor-β (TGF-β) is a secreted protein that regulates proliferation, differentiation and death in various cell types including thyroid cells, although few details are known about its mechanisms of action in this cell type. Here we studied the role of TGF-β on the regulation of the sodium/iodide symporter (NIS) gene expression in PC Cl3 thyroid cells. TGF-β inhibits thyroid-stimulated hormone (TSH)-induced NIS mRNA and protein levels in a dose-dependent manner. This effect takes place at the transcriptional level, as TGF-β inhibits TSH-induced transcription of a luciferase reporter construct containing a 2.8 kb DNA fragment of the rat NIS promoter. The inhibitory effect of TGF-β was partially overcome by inhibitory Smad7 and mimicked by overexpression of either Smad3 or a constitutively activated mutant of TGF-β-receptor I (TβRI) (acALK-5). Using internal deletions of the promoter, we define a region between −2,841 to −1,941, which includes the NIS upstream enhancer (NUE), as responsible for the TGF-β/Smad inhibitory effect. NUE contains two binding sites for the paired-domain transcription factor Pax8, the main factor controlling NIS transcription. The physical interaction observed between Pax8 and Smad3 appears to be responsible for the decrease in Pax8 binding to DNA. Expression of Pax8 mRNA and protein was also decreased by TGF-β treatment. The results suggest that through activation of Smad3, TGF-β decreases Pax8 DNA-binding activity as well as Pax8 mRNA and protein levels, which are at least partially involved in TGF-β-induced downregulation of NIS gene expression in thyroid follicular cells. Our results thus demonstrate a novel mechanism of Smad3 function in regulating thyroid cell differentiation by functionally antagonizing the action of the paired-domain transcription factor Pax8.
INTRODUCTION

Iodide is an essential element in thyroid physiology, as a critical component of thyroxine and triiodothyronine molecules, and a key regulator of thyroid gland function. The first step in iodide metabolism is represented by thyroid trapping, which is achieved by an active, energy-dependent transport process across the basolateral plasma membrane of the thyrocytes. The protein responsible for this process, the sodium/iodide symporter (NIS), is an intrinsic plasma membrane protein that mediates active transport of I\(^-\) in the thyroid, lactating mammary gland, stomach and salivary glands (1-4). NIS plays key role in thyroid pathophysiology as the route by which iodide reaches the gland for thyroid hormone biosynthesis, and as means for diagnostic scintigraphic imaging and for radioidide therapy in hyperthyroidism and thyroid cancer (2,4).

The molecular characterization of NIS started with the cloning of the cDNA encoding rat NIS in 1996 (5). The rNIS gene has a minimal promoter between -199 and -110 bp (3,6-8) and an upstream enhancer, NUE (NIS upstream enhancer), between –2495 and –2264 bp (9). The NUE stimulates transcription in a thyroid-specific, cAMP-dependent manner and involves the most relevant aspect of NIS regulation. NUE contains TTF-1 binding sites that have no effect on NIS transcription, Pax8 binding sites and a degenerate CRE (cAMP responsive element) sequence. Full TSH-cAMP-dependent transcription requires Pax8 binding and the integrity of the CRE-like sequence in rat and in human NIS promoter (3,9,10).

Pax8, one of the thyroid transcription factors, is a member of the murine Pax family of paired-box-containing genes, expressed in the developing kidney, the neural tube, and the developing and adult thyroid (11-13). Pax8 has an essential role in thyroid organogenesis and in differentiation being the main mediator of thyroid genes transcription (13,14), including the NIS gene (9,15).
I\(^-\) uptake is stimulated by TSH, the master hormone for thyroid gland regulation. TSH stimulation results, at least in part, from the cAMP-mediated increase in NIS biosynthesis. TSH not only stimulates NIS transcription and biosynthesis, but is also required for modulating the NIS phosphorylation pattern, maintaining its half life, and retaining NIS at the thyrocyte plasma membrane (4,16,17). The main factor regulating NIS activity in the thyroid, other than TSH, has long been considered to be iodide itself. In addition to TSH and I\(^-\), cytokines, estrogen, growth factors, thyroglobulin (Tg), and dexamethasone also play a role in NIS modulation (3,4,18-21). Both infiltrating inflammatory cells and thyroid follicular cells themselves produces cytokines, which affect thyroid function and growth, and cause immunological changes in the gland. The cytokines studied include TGF-\(\beta\), TNF-\(\alpha\) and \(\beta\), INF-\(\gamma\), and IL, all of which exert an inhibitory effect on thyroid function, including decreased NIS expression and I\(^-\) uptake (4,18,19,22).

The TGF-\(\beta\) family of cytokines regulates cell proliferation, survival, adhesion, migration, differentiation and specification of developmental fate. TGF-\(\beta\) is also a potent immunosuppressor, and perturbation of its signaling is linked to autoimmunity, inflammation and cancer (23-27). The Smads are the family of intracellular transducers that act downstream of receptors for TGF-\(\beta\) family members, and they are the only TGF-\(\beta\)-receptor substrates with demonstrated ability to propagate signals (23-25,28,29). TGF-\(\beta\) binds to a specific serine/threonine kinase type II receptor on the cell surface, resulting in activation of the dormant kinase activity of type I receptor. The activated receptor then phosphorylates members of the Smad family, termed R-Smads (Smad2 and Smad3). The phosphorylated R-Smads form oligomers with the unique Co-Smad (Smad4), and rapidly translocate to the nucleus where the Smad complex participates in transcriptional regulation. A third class of Smads, inhibitory
Smads (Smad7), antagonizes R-Smad activity. Smads thus signal transiently in the nucleus by controlling expression of specific genes (23-25,28,29).

TGF-β production and secretion, as well as the expression of TGF-β-specific receptors, have been demonstrated in normal and diseased thyroid follicular cells (30,31), and several TGF-β family members have been implicated in the regulation of thyroid growth and function. Although several reports have shown that TGF-β regulates thyroid-specific genes (4,18,31-36), the mechanism by which TGF-β modulates thyroid function remains unknown.

Here we studied NIS gene regulation by TGF-β, and further explored the mechanism of TGF-β action by examining the involvement of Smad pathway in the thyroid. We show that TGF-β decreases NIS gene expression in PC Cl3 cells by reducing TSH-transcriptional activation. A responsive region within the NIS promoter (-2841/-1941), containing two Pax8 binding sites, is necessary for the TGF-β response. In addition, we provide evidence of a novel repression mechanism involving a physical and functional interaction between Pax8 and Smad3. This interaction may explain the mechanism of TGF-β downregulation of NIS gene expression in thyroid follicular cells, and might be extended to other genes transactivated by Pax8.

EXPERIMENTAL PROCEDURES

Cell Culture-PC Cl3 cells were cultured in Coon’s modified Ham’s F-12 medium supplemented with 5% donor calf serum and six-hormone mixture (1 nM TSH, 10 µg/ml insulin, 10 ng/ml somatostatin, 5 µg/ml transferrin, 10 nM hydrocortisone, and 10 ng/ml glycy1-L-histidyl-L-lysine acetate; complete medium). The effect of hormones and growth factors was studied by starving near-confluent cells for TSH and insulin in the presence of 0.2 % serum (starvation medium) for 72 h. Due to the exceptionally long half-life of NIS (4), to analyze this
protein cells were starved for 7 days. After starvation, TSH (1 nM) and TGF-β (10 ng/ml) were added to culture medium (24 h unless otherwise indicated). Hormones were obtained from Sigma and TGF-β from PeproTech.

**RNA Extraction and Northern Blot Analysis**—Total RNA was isolated by the guanidinium-thiocyanate-phenol procedure (37) from PC Cl3 cells after treatments. Total RNA (20 µg) was separated in 1% agarose gels containing 0.44 M formaldehyde. RNA was blotted onto Nytran filters (Schleicher & Schuell). Hybridization was carried out with the specific and 18S rRNA probes, labeled by random oligo priming.

**Protein Extraction and Western Blot Analysis**—Membranes from PC Cl3 cells were prepared by collecting cells in a buffer containing 10 mM Hapes-KOH pH 7.5, 250 mM sucrose, 1 mM EDTA, 2 µg/ml leupeptin, and 2 µg/ml aprotinin. Cells homogenates were centrifuged (100,000 x g, 60 min, 4°C) and the pellet was resuspended in buffer as above. For subcellular fractionation, PC Cl3 cells were collected in buffer A (10 mM Hapes-KOH pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) and sedimented at low centrifugal force to pellet nuclei and unbroken cells. The supernatant (cytoplasmic extract) was collected and the nuclear pellet resuspended in buffer C (20 mM Hapes-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) (38). Cellular debris was removed by centrifugation and the supernatant fraction was collected. Nuclear, cytoplasmic and membrane proteins concentration were determined according to Bradford (39). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane (Schleicher & Schuell). Ponceau S staining of the blots for NIS detection showed equal protein loading. Membranes were blocked and incubated with the antibodies. Immunoreactive bands were visualized with Luminol Western blot detection reagent (Santa Cruz Biotechnology).
Plasmids—The 2,854 bp DNA fragment (pNIS-2.8) of the rat NIS promoter was cloned in our laboratory (21). The 5′-deletion constructs with (pNIS-NUE-1.2 and pNIS-NUE-0.5) or without NUE (pNIS-2, pNIS-1.2 and pNIS-0.5) were previously described (21). pRL-TK, which contains a cDNA encoding Renilla (Promega), was used to monitor transfection efficiency. The expression vectors pCMV5-FLAG-Smad3, pCMV5-Smad4-HA, pcDNA3-FLAG-Smad7 encoding human Smad members FLAG or hemagglutinin epitope tagged and pCMV5-TβRI (ALK-5) harboring a constitutively activated form of the TGF-β receptor type I (TβRI), have been previously described (40-42). Full length Pax8 and the carboxy-terminal deletion ΔPax8 were subcloned in our laboratory in the pcDNA3.1+ vector.

Transfection—PC Cl3 cells were plated at 6 x 10⁵ cells per 60 mm diameter tissue culture dish, 48 h before transfection. Transfections were performed by calcium phosphate coprecipitation. To study the effect of TSH and TGF-β, transfected cells were cultured in starvation medium (72 h), and treated with TSH or TSH plus TGF-β. After 24 h, cells were collected for LUC and Renilla activity assay using Dual-Luciferase Reporter Assay System (Promega). In co-transfection experiments, the amount of DNA was normalized using the corresponding insertless expression vector as carrier.

GST Pull-down Analysis—For GST pull-downs, GST fusion constructs of Smad3 or Pax8 were expressed in *Escherichia coli* and purified using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). Equal amounts of GST or GST-Smad3 bound to glutathione Sepharose beads were incubated with *in vitro*-transcribed and translated (TNT translation kit, Promega) full-length Pax8 or ΔPax8 labeled with (³⁵S)-Met on ice for 2 h. Beads were washed five times in wash buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10% glycerol and protease inhibitors), and specifically-bound proteins were detected by SDS-
TGF-β/Smad3 represses NIS gene expression

PAGE and autoradiography. In GST pull-down assays with nuclear cell extracts, PC Cl3 cell extracts were incubated with GST or GST-fusion proteins (GST-Smad3 and GST-Pax8) bound to glutathione Sepharose beads, on ice for 2 h. Beads were washed in wash buffer and specifically-bound proteins detected by Western blot analysis. Specific immunodetection was carried out by incubation with anti-Pax8 or anti-Smad3 antibodies (Santa Cruz Biotechnology).

Band-Shift Assay-Band-shift probe corresponding to an oligonucleotide of Pax8 binding sites in rNIS promoter (site PB within the NUE) (9) was labeled with 32P by polynucleotide kinase. Nuclear extracts from PC Cl3 cells (7 µg), recombinant Pax8 (TNT-Pax8) or bacterially expressed GST and GST-Smad3 proteins were incubated with the labeled probe. Binding reactions were performed in a buffer containing 40 mM Hepes pH 7.9, 75 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 150 ng/µl poly-(dI-dC), and 5% Ficoll, at room temperature for 30 min. When required, samples were supplemented with an anti-Pax8 antibody from Santa Cruz Biotechnology (F-19) or provided by Dr. R. Di Lauro (9). Samples were electrophoresed on a 5% polyacrylamide gel in 0.5 x TBE. For competition, an excess of cold oligonucleotide was added to the reaction mixture.

RESULTS

TGF-β Inhibits TSH-Induced NIS mRNA and Protein Levels-Starved PC Cl3 cells were treated with TSH or TSH plus TGF-β, and NIS mRNA and protein levels were analyzed by Northern (A) and Western blot (B) assays. As described (9), TSH stimulated NIS mRNA (Fig. 1A) and protein (Fig. 1B) levels and TGF-β repressed the stimulatory effect of TSH in a dose-dependent manner (A and B). The maximum inhibitory effect was reached at 10 ng/ml of TGF-β. These data demonstrated that TGF-β interferes with TSH induction of NIS gene expression.
TGF-β Downregulates TSH-Dependent Transcriptional Activation of the NIS Gene—To study whether TGF-β regulates transcription of the NIS gene, we used a 2.8 kb fragment of the rat NIS promoter (pNIS-2.8)(21). The pNIS-2.8 contains the NUE, a regulatory element necessary for a full TSH response (9). Luciferase reporter constructs containing the full-length DNA fragment or 5’-deletion derivatives were transiently transfected into PC Cl3 cells and assayed for transcriptional activity in response to TSH and TGF-β. TSH induced a significant stimulation of pNIS-2.8 activity, whereas TGF-β decreased the TSH-mediated transactivation in a dose-dependent manner (Fig. 2A). The NIS promoter activity was not affected by TGF-β in the absence of TSH (not shown).

To identify the promoter region responsible for TGF-β regulation, we analyzed the transcriptional activity of NUE-less 5’-deletion constructs pNIS-2, pNIS-1.2 and pNIS-0.5 (Fig. 2B). As previously described, the NUE is required for full TSH stimulation of the NIS gene, and TGF-β had no effect on the transcriptional activity of any 5’-deletion constructs (Fig. 2B). TGF-β inhibition was thus abolished in chimeras that do not bear the NUE enhancer. The transfection of the constructs pNIS-2.8, pNIS-NUE-1.2 and pNIS-NUE-0.5 showed a significant activity in response to TSH, as well as a reduction in luciferase activity when TGF-β was added together with TSH for 24 h (Fig. 2C).

In summary, the results indicate that TGF-β interferes with TSH-dependent transcriptional activation of the NIS gene, and that TGF-β requires elements within or adjacent to the NUE for this regulation.

NIS Gene Repression by TGF-β is Signaled by the Smad Proteins—To analyze the mechanism by which TGF-β downregulates TSH-induced NIS gene transcription, we studied the intracellular mediators of the TGF-β pathway. A signaling cascade is initiated after TGF-β
TGF-β/Smad3 represses NIS gene expression

binding, resulting in R-Smad activation, oligomerization with Smad4, and subsequent translocation of the Smad complex to the nucleus, where it participates in regulation of gene transcription (23,28). To assess Smad involvement in the TGF-β effect, we co-transfected the rNIS promoter construct (pNIS-2.8) with expression vectors harboring Smad3 or Smad4 (Fig. 3A). TSH-stimulated NIS promoter activity was decreased by overexpression of Smad3 and Smad4. As predicted, the strongest inhibition was observed when both Smads were expressed (Fig. 3A). The inhibition induced by Smad4 might be caused by interaction with endogenous Smad3. Overexpression of Smad proteins thus mimicked the inhibitory effect of TGF-β on NIS gene transcription shown above, supporting a role for the Smad pathway in TGF-β action. As Smad2 overexpression did not reproduce the TGF-β effect on NIS promoter activity (not shown), we discarded its mediation in TGF-β transcriptional repression.

To confirm the participation of Smad signaling in TGF-β regulation of NIS, we transfected PC Cl3 cells with pNIS-2.8 in the presence of the constitutively activated TGF-β-receptor type I (TβRI) (acALK-5) that phosphorylates and activates TGF-β-dependent R-Smads (24,29). Through activation of endogenous Smads, acALK-5 overexpression reduced TSH-stimulated NIS gene transcription, mimicking the effect of TGF-β (Fig. 3B). acALK-5-mediated inhibition was dependent on the amount of vector expressed in the cell and was similar to that exerted by TGF-β.

We also analyzed the transcriptional activity of the NIS promoter (pNIS-2.8) in the presence of an inhibitory Smad (Smad7). Smad7 forms a stable association with the activated type I receptors, preventing phosphorylation of R-Smads and blocking downstream TGF-β signaling (42). Transient overexpression of Smad7 in PC Cl3 cells partially abrogated repression of NIS promoter activity by TGF-β (Fig. 3C). At the same concentrations, Smad7 partially
TGF-β/Smad3 represses NIS gene expression

prevented inhibition of the NIS promoter construct by acALK-5 (Fig. 3D), indicating the specificity of Smad7 in blocking Smad-mediated transcriptional repression.

All together, the results firmly establish the functional requirement for Smad3 in mediating TGF-β transcriptional repression.

Nuclear Accumulation of Smad3 and Smad4 by TGF-β—Smad3 and Smad4 normally reside in the cytoplasm of unstimulated cells and are imported to the nucleus as complexes in response to TGF-β signaling to regulate target gene transcription (23-25,28,29). Since the nuclear entry of the Smads is a key step in signal transduction, we analyzed the accumulation of Smad3 and Smad4 in the nucleus of PC Cl3 cells after TGF-β treatment. We observed a rapid and a striking increase in both proteins in the nuclear fraction (Fig. 4A), with a parallel decrease in the cytosolic fraction (Fig. 4B). This effect is TGF-β-specific, since TSH alone did not induce Smad translocation to the nucleus (compare first and second lines of Fig. 4A, B). After prolonged TGF-β treatment (4 and 24 h), Smad3 and Smad4 levels in nuclear extracts fall, although a corresponding increase was not detected in the cytoplasmic fraction. This effect could be explained by the previously described degradation of Smads induced after long treatment with TGF-β (23).

Our results showed accumulation of both Smads in the PC Cl3 cell nucleus, confirming activation of the Smad signaling pathway by TGF-β and supporting Smad participation as intracellular effectors of TGF-β action in thyroid follicular cells.

TGF-β Decreases the Binding of Pax8 to its Response Element in NIS Promote-The NUE has two Pax8 binding sites, which are required to obtain the full TSH/cAMP-dependent transcription (4,9). Here we demonstrate that TGF-β requires the NUE to decrease the TSH-dependent transcriptional activation of NIS. These observations prompted us to analyze whether
TGF-β modified the ability of Pax8 to bind DNA. For the assay we used a radiolabeled synthetic oligonucleotide probe spanning -2409 to -2377 bp of the rNIS promoter, Oligo PB (9) (see Fig. 5 upper panel). The band-shift assay showed that TSH stimulates Pax8 binding to NUE (Fig. 5, lane 7) compared with unstimulated cells (lane 6). These data showed a reduction in the intensity of the Pax8/DNA complex in TGF-β-treated cells (lanes 8-13) compared to the control (lane 7). Complex specificity was confirmed by competition with the related (lane 3) but not an unrelated oligonucleotide (lane 2). It is also displaced by an antibody that recognizes a Pax8 DNA-binding domain epitope (αPax8 (F-19)) (lane 4). A supershift was observed with another αPax8 antibody raised against a peptide mapping downstream of the paired domain (lane 5). The same TGF-β regulation was observed when the PA oligonucleotide, containing the other NUE-Pax8 binding site (see Fig. 5 upper panel), was used (not shown). The results indicate that TGF-β alters Pax8 binding to the NIS promoter, an event that could explain TGF-β inhibition of NIS promoter activity (Fig. 2).

Although Smad-binding elements (SBE) are often present in the responsive region of TGF-β target genes (23,28,29), we could not attribute the modulation of NIS gene transcription to SBE, since we did not observe binding of activated Smads to SBE motifs in the NIS promoter (not shown). On the other hand, we never found binding of recombinant Smad proteins (GST-Smad3 and GST-Smad4) to the Pax8-binding elements in the NIS promoter (not shown).

*Physical Interaction Between Pax8 and Smad3*- Smad proteins regulate transcription in collaboration with other transcription factors through direct protein-protein interactions (25,28). We thus studied a potential physical interaction between Smad3 and Pax8 using GST-pull-down assays. ΔPax8 is a carboxy-terminal deletion of Pax8, which includes the DNA-binding domain and lacks the transactivation domain (see Fig. 6A). Both recombinant full-length Pax8 and
ΔPax8 form complexes with GST-Smad3 linked to Sepharose beads (Fig. 6A, lanes 3, 6), but not with GST-beads in a cell-free system (lanes 2, 5). The observation that the truncated Pax8 binds the GST-Smad3 (lane 6) indicates that the physical interaction between Smad3 and Pax8 proteins does not involve the Pax8 activation domain, whereas the amino-terminal end of Pax8 appears to be sufficient for this interaction.

In a second experimental approach, physical interactions between Pax8 and Smad3 were also demonstrated in vitro using GST-pull-down assay with nuclear PC Cl3 cell extracts (Fig. 6B). Specifically, fusion proteins consisting of GST-Smad3, GST-Pax8 or the GST portion alone coupled to Sepharose beads were incubated with nuclear extracts of quiescent PC Cl3 cells (lanes 1, 7) or cells treated with TSH plus TGF-β (lanes 2, 8). Interaction between Smad3 and Pax8 was confirmed through the complexes formed with GST-Smad3 beads and endogenous Pax8 (lanes 5, 6), as well as with GST-Pax8 beads and endogenous Smad3 (lanes 11, 12). No interaction was found with GST-beads (lanes 3-4 and 9-10).

**DNA Binding Activity of Pax8 to NIS Promoter is Reduced by Smad3**—Here we demonstrated that TGF-β decreases Pax8 DNA binding to NIS promoter, and also found a physical interaction between Pax8 and Smad3. We studied the possible role of Smad3 in regulation of Pax8/DNA complex using recombinant Smad3 (GST-Smad3). The band-shift assay performing with nuclear extract from PC Cl3 cells (Fig. 7A) or recombinant Pax8 (TNT-Pax8) (Fig. 7B), showed that as the GST-Smad3 concentration increased (Fig. 7 A,B lanes 5-9), the amount of Pax8/DNA complex decreased. No effect was found with GST-alone (Fig. 7 A,B lanes 2-4).

These findings indicate that Smad3 is able to inhibit Pax8/DNA complex dependent upon the association of Smad3 with Pax8-paired domain.
*TGF-β Decreases mRNA and Protein Levels of Pax8 in PC Cl3 Cells*- We also studied expression of Pax8 mRNA and protein in response to TGF-β. The results demonstrated that TGF-β downregulated TSH-stimulated Pax8 mRNA (Fig. 8A) and protein (Fig. 8B) levels. As predicted, the decrease in Pax8 mRNA levels (A) preceded the downregulation of Pax8-protein expression (B).

All together, the results indicate that TGF-β interferes with Pax8 transcriptional activation of the NIS promoter by two mechanisms that include an initial physical interaction between Pax8 and Smad3 and later negative regulation of Pax8 expression.

**DISCUSSION**

The sodium/iodide symporter mediates the active transport of iodide in thyroid follicular cells (1-5). A number of agents regulate NIS expression; among these, TGF-β is a potent inhibitor of both iodide uptake (3,4,43-45) and NIS gene expression (3,4,18,45). Similarly, TGF-β reduces the activity and the mRNA level of the Na⁺/K⁺ ATPase in a time- and dose-dependent manner in FRTL-5 cells (22). When FRTL-5 cells aged, an increase in TGF-β expression and secretion was observed, in turn decreasing NIS mRNA levels and I⁻ transport (18).

Here we confirmed the previously demonstrated dose-dependent inhibition of NIS mRNA levels by TGF-β (18,22,45), and demonstrated reduced NIS protein expression, also dependent on TGF-β dose. The inhibition we observed, as well as that reported by others (18,45), was relative to the TSH-stimulated levels of NIS. At present, it is thought that the potent suppression of thyroid growth and differentiation elicited by TGF-β modulates the effects of TSH and other growth factors to maintain thyroid gland homeostasis (46). In addition to NIS, TGF-β acts as a potent suppressor of other thyroid-restricted genes such as Tg, TPO and TSHR (31,35,44,45),
again suggesting that TGF-β modulates the TSH-stimulated effect on these genes (45). As TGF-β is produced in normal and diseased thyroid gland (30,32), this cytokine might participate in an auto-regulatory mechanism that counterbalances TSH-stimulated action on thyrocytes in physiological conditions, and alterations in this pathway could be responsible for pathological processes.

Although the inhibitory function of TGF-β on thyroid cell growth, differentiation, and gene regulation is well documented, there is little information regarding its precise mechanisms of action in this tissue. We showed that TGF-β-induced inhibition of NIS transcription occurs through activation of TGFβ-specific receptors, due to Smad3 accumulation in thyrocyte nuclei, with a consequent decrease in the cytoplasmic fraction. The presence of TGF-β-specific receptors I and II, as well as their activation by the ligand, have been demonstrated at thyrocyte membrane (31). We subsequently examined Smad involvement as TGF-β-dependent effectors in NIS transcription. Studies with Smad3 and Smad4 expression vectors indicated a role for Smad3 protein as the mediator of TGF-β-induced NIS transcriptional repression. These observations are supported by cotransfection with the constitutively active form of the TGF-β receptor I (acALK-5), which activates endogenous Smad, repressing TSH-activated NIS gene transcription and mimicking TGF-β action on this gene. The participation of Smad as a downstream effector in TGF-β transcriptional repression was also confirmed by overexpression of inhibitory Smad7 (28), which abrogated part of the transcriptional repression mediated by TGF-β and by acALK-5. We demonstrated not only the presence of Smad3 and Smad4 in PC Cl3 cells, but also their activation and nuclear accumulation after TGF-β treatment confirming data in porcine thyroid cells (31). In support with our findings is the fact that Smad3 mediates TGF-β-dependent inhibition of a number of genes (29,35,47,48).
To analyze the role of TGF-β in the regulation of NIS gene transcription, we used the 2.8-kb DNA fragment of the rat NIS promoter (21). Results of transient transfection assays confirm the requirement for the NIS upstream enhancer (NUE) for a potent TSH response (9). Moreover, we showed that TGF-β represses TSH transcriptional activation in a dose-dependent manner. To delimit the region responsible for TGF-β regulation, we studied several deletions of the NIS promoter. The region between -2841 to -1941 bp, which includes the NUE, is responsible for the TGF-β/Smad3 inhibitory effect. These results indicate that TGF-β interferes with TSH activation of NUE, an important enhancer that contains Pax8-binding sites essential for transactivation of NIS (3,4,9).

The increase in Pax8 mRNA and protein levels, as well as the increase in the DNA binding elicited by TSH, agrees with previous work defining Pax8 as the main mediator of the TSH-dependent gene activation in thyroid follicular cells (49). We thus hypothesized that Pax8 could be the target of TGF-β/Smad3 inhibition on the NIS gene, and performed band-shift assays to elucidate whether Pax8 participates in this mechanism. The results clearly show the ability of TGF-β to alter the DNA binding activity of Pax8. The effect was observed 15 min after TGF-β treatment and coincides with the accumulation of Smad3 in the nucleus.

Here we define a novel mechanism of NIS transcriptional repression that involves a protein-protein interaction between Pax8 and Smad3. Since the interaction between these two proteins includes the DNA-binding domain of Pax8, Smad3 may interfere with NIS transcription by impairing Pax8 binding, rather than its transactivation. The results may explain the decrease in Pax8/DNA complexes found after TGF-β treatment. The evidence suggests that Pax8 is required for inhibition of TGF-β/Smad3-dependent transcription.
In the nucleus, Smad proteins bind DNA very weakly, and must be recruited to DNA by other transcription factors (24,29). Few studies have successfully demonstrated Smad3/DNA binding (50). Various transcription factors recruit Smads through protein-protein interaction to specific promoters, and the binding modified the activity of an existing transcriptional complex (23,24). Since we could not demonstrate direct Smad3 binding to the NIS promoter (not shown), the functional interaction between Pax8 and Smad3 does not depend on the presence of DNA consensus sequences. Different reports found that Pax8 is able to recruits transcription factors (15,51) or coactivators (52) to the promoter of target genes; thus Pax8 may have a role in the recruitment of Smad3 to NIS promoter, resulting in a functional interaction between these proteins. A similar model of repression has been observed with Pax 2/5/8 family members and HLH Id proteins where the interaction between them involves the paired domain of Pax 2/5/8 proteins, and resulted in the disruption of the DNA-bound complexes (53). Here we demonstrated a decrease in Pax8 DNA-binding activity as well as a later downregulation in Pax8 mRNA and protein levels induced by TGF-β treatment.

All transformed thyroid cells with either a high or low degree of malignancy display a loss of iodide uptake. To achieve NIS maximal expression in thyroid cancer for radiiodine treatment, it would be necessary not only to stimulate NIS expression with endogenous or exogenous TSH, but also to eliminate NIS inhibitors such as TGF-β.

In summary, the increase in Smad3 protein in the PC Cl3 cell nucleus in response to TGF-β interferes with Pax8 transcriptional activation of the NIS gene through mechanisms involving rapid physical interaction and long-term downregulation of the Pax8 expression. Since Pax8 is important for TSH-dependent transcriptional activation, TGF-β/Smad signal prevents the Pax8-mediated NIS gene transactivation.
We have demonstrated physical and functional interaction between Smad3 and the Pax8 transcription factor. This interaction impairs transcription by altering the conformation of the DNA-binding complex, and has implications for several important cellular processes that may contribute to physiological and pathological conditions of the thyroid gland.

Acknowledgements-We are indebted to Dr. A. Fusco (Universita degli Studi di Napoli, Naples, Italy) for providing PC Cl3 cells, Dr. C.H. Heldin (Ludwig Institute for Cancer Research, Sweden) for pcDNA3-FLAG-Smad7, Dr. C. Bernabeu (CIB, CSIC, Madrid, Spain) for pCMV5-FLAG-Smad3, pCMV5-Smad4-HA and GST-Smad3, Dr. M. Rivas (IIB. CSIC, Madrid, Spain) for the pcDNA3.1+Pax8 and pcDNA3.1+ΔPax8 constructs, Dr. R. Di Lauro (Stazione Zoologic, Naples, Italy) for providing Pax8 cDNA and Pax8 antibody, Dr. N. Carrasco (Albert Einstein College of Medicine, Bronx, NY) for the NIS cDNA and the NIS antibody. We thank Catherine Mark for her linguistic assistance.

This study was supported by grants BMC 2001-2087 from MCYT (Spain), CAM 08.2/0025/97 and FIS of the Instituto de Salud Carlos III, RCMN (C03/08) and RGDM (G03/212).

EC is a postdoctoral fellow of the Fundación Carolina (Spain). BG is a postdoctoral fellow of the CAM (Spain).

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TGF-β/Smad3 represses NIS gene expression

FIGURE LEGENDS

FIG. 1. **TGF-β inhibits TSH-induced NIS mRNA and protein levels.** Starved PC Cl3 cells were treated with TSH or TSH plus TGF-β (1-10 ng/ml) for 24 h, then harvested for RNA extraction (A) or membrane proteins preparation (B). A, A representative Northern blot hybridized with rNIS probe. As a loading control, the same membrane was hybridized with 18S rRNA probe. B, A representative Western blot of membrane proteins (5 µg) was probed with anti-rNIS antibody. As a loading control, Ponceau S staining of a blot fragment is shown.

FIG. 2. **TGF-β downregulates TSH-dependent transcriptional activation of the NIS gene.** PC Cl3 cells were transfected with 250 ng of pRL-TK and 4 µg of the reporter construct containing the complete rNIS promoter, pNIS-2.8 (panel A), 5’-deletion derivatives pNIS-2.0, pNIS-1.2, pNIS-0.5 (B), or internal deletion constructs pNIS-NUE-1.2 or pNIS-NUE-0.5 (C). After transfection, cells were maintained 72 h in starvation medium, and treated with TSH or TSH plus TGF-β (10 ng/ml except in A) for 24 h. Relative luciferase activity is the value of x-fold induction over the value of starved cells (without TSH). The data represent the mean ± SD of one experiment representative of at least three independent experiments.

FIG. 3. **NIS gene repression by TGF-β is signaled by the Smad proteins.** A, Repression of NIS promoter activity by Smad3 and Smad4: PC Cl3 cells were cotransfected with 3 µg of the reporter construct pNIS-2.8, 250 ng of pRL-TK and either 1 µg of Smad3 and/or Smad4 expression vectors. After transfection, cells were maintained 72 h in starvation medium, then treated with TSH for 24 h. B, Transcriptional repression of the NIS promoter by TβRI (acALK-5): PC Cl3 cells were cotransfected with the pNIS-2.8 promoter construct (3 µg), 250 ng of pRL-TK and acALK-5 expression vector (0.5, 1, 2 µg). After transfection, cells were maintained 72 h
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in starvation medium and then treated with TSH or TSH plus TGF-β (10 ng/ml) for 24 h. C, and D, Smad7 partially abrogates the downregulation of NIS promoter activity mediated by TGF-β (C) or by acALK-5 (D). PC Cl3 cells were cotransfected with 3 µg of pNIS-2.8, 250 ng pRL-TK, and Smad7 expression vector (0.75, 1, 1.5 µg), alone (C) or in the presence of acALK-5 (1 µg) (D). After transfection, cells were treated as in B. A-D, NIS promoter activity is expressed as x-fold induction over the control value (+TSH). The data represent the mean ± SD of one representative of at least three independent experiments.

FIG. 4. **Nuclear accumulation of Smad3 and Smad4 by TGF-β.** Starved PC Cl3 cells were treated for 24 h with TSH plus TGF-β or with TSH alone, then with TSH plus TGF-β for different times (1/4-4 h). Cells were then harvested for nuclear or cytosolic extract preparation. Representative Western blots are shown for nuclear extract (15 µg) (A) and cytoplasmic extract (35 µg) (B) hybridized with anti-Smad3 and anti-Smad4 antibodies. As a loading control, the membranes were hybridized with αSp1 (A) or αactin (B) antibodies.

FIG. 5. **TGF-β decreases the binding of Pax8 to its response element in NIS promoter.**

Upper panel, Schematic representation of the rNIS promoter structure. Lower panel, Starved PC Cl3 cells were treated for 24 h with TSH plus TGF-β or with TSH alone, then with TSH plus TGF-β for different times (1/4-4 h). Cells were then harvested for nuclear extract preparation. Band-shift assay was performed with a 32P-labeled oligonucleotide corresponding to the Pax8 binding site (sites PB) derived from the NIS promoter. The probe was incubated without extracts (lane 1) or with nuclear extracts from PC Cl3 cells (7 µg) (lanes 2-13), in the presence (+) or absence of αPax8 antibodies (lanes 4 and 5). For competition, a 100-fold excess of oligonucleotide PB (R) (lane 3) or unrelated (NR) (lane 2) cold oligonucleotide was used.
FIG. 6. **Physical interaction between Pax8 and Smad3.**  

A, *Upper panel,* Schematic representation of full-length Pax8 and ΔPax8 constructs. Locations are indicated of the N-terminal paired-box DNA-binding (PD), repressor (RD) and activating (AD) domains. *Lower panel,* GST pull-down assay in a cell-free system to demonstrate Pax8 and ΔPax8 interaction with Smad3.  

35S-labeled Pax8 (lanes 1-3) or ΔPax8 (lanes 4-6) were incubated *in vitro* with Sepharose-bound GST (lanes 2 and 5) or GST-Smad3 (lanes 3 and 6), and interacting protein visualized by SDS-PAGE and autoradiography. A fraction of input proteins (20%) was analyzed for comparison (lanes 1 and 4).  

B, Pax8 interaction with Smad3 in a GST pull-down assay with nuclear proteins. Nuclear extracts from quiescent PC Cl3 cells (lanes 1 and 7) or treated with TSH (24 h) plus TGF-β (1 h) (lanes 2 and 8) PC Cl3 cells were incubated with GST- or the indicated GST-fusion protein-coupled Sepharose beads. Bound proteins were analyzed by SDS-PAGE, and Western blot was performed using anti-Smad3 and anti-Pax8 antibodies. Input represents 30% of the initial nuclear cell extract used in the binding experiments. WB: Western blot.

FIG. 7. **DNA-binding activity of Pax8 to NIS promoter is reduced by Smad3.**  

Starved PC Cl3 cells were treated with TSH for 24 h, then harvested for nuclear extract preparation (*A*). Band shift assays were performed with 32P-labeled oligonucleotide corresponding to the Pax8 binding site (PB site) within the NIS promoter incubated with nuclear extract (5 µg) (*A*) or with recombinant Pax8 (TNT-Pax8) (*B*) in the absence (lane 1) or presence of increasing amounts of GST (lanes 2-4), or GST-Smad3 (lanes 5-9).

FIG. 8. **TGF-β decreases Pax8 mRNA and protein levels.** Starved PC Cl3 cells were treated for 24 h with TSH plus TGF-β or with TSH alone, then with TSH plus TGF-β for different times (1/4-4 h). Cells were then harvested for RNA extraction (*A*) or nuclear extracts preparation (*B*).
A, A representative Northern blot hybridized with Pax8 probe is shown. As a loading control, the membrane was hybridized with 18S rRNA probe. B, A representative Western blot of nuclear extract (15 µg) was probed with αPax8 antibody. As a loading control, the membrane was hybridized with αSp1 antibody.
Figure 1

A

<table>
<thead>
<tr>
<th>TGF-β (ng/ml)</th>
<th>0</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSH</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

NIS mRNA

2.8 Kb

18 S

B

<table>
<thead>
<tr>
<th>TGF-β (ng/ml)</th>
<th>0</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSH</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

NIS protein

111

80

61

49
Figure 2

A

Relative luciferase activity (fold induction)

TGF-β (ng/ml)

B

Relative luciferase activity (fold induction)

C

Relative luciferase activity (fold induction)

-TSH  +TSH  TSH+TGF-β
Figure 3
Figure 4

A  Nuclear extract  
TGF-β (h)  0  1/4  1/2  1  2  4  24  
Smad3  
Smad4  
Sp1

B  Cytoplasmic extract  
TGF-β (h)  0  1/4  1/2  1  2  4  24  
Smad3  
Smad4  
Actin
TGF-β (h) 0 0 0 0 0 0 1/4 1/2 1 2 4 24
TSH - - + + - + + + + + +

Supershift →
Pax8/DNA complex →

DNA free

1 2 3 4 5 6 7 8 9 10 11 12 13

Oligo R +
Oligo NR +
αPax8 (F-19) +
αPax8 +

Figure 5
Figure 6

A

\[
\begin{align*}
\text{Pax8} \quad \text{NH}_2 & \quad = \quad \text{PD} \quad \text{RD} \quad \text{AD} \quad = \quad \text{COOH} \\
\Delta \text{Pax8} \quad \text{NH}_2 & \quad = \quad \text{PD} \quad \text{AD} \quad = \quad \text{COOH}
\end{align*}
\]

B

\[
\begin{align*}
\text{TGF-}\beta & \quad - \quad + \quad - \quad + \quad - \quad + \\
\text{TSH} & \quad - \quad + \quad - \quad + \quad - \quad + \\
\text{WB: Pax8} & \quad \text{Input 30\%} \quad \text{GST} \quad \text{GST-Smad3} \\
\text{Input 30\%} & \quad 1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \\
\text{WB: Smad3} & \quad \text{Input 30\%} \quad \text{GST} \quad \text{GST-Pax8} \\
\text{Input 30\%} & \quad 7 \quad 8 \quad 9 \quad 10 \quad 11 \quad 12
\end{align*}
\]
Figure 8
The functional interaction between the paired-domain transcription factor Pax8 and Smad3 is involved in the TGF-b repression of the sodium iodide symporter gene

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J. Biol. Chem. published online November 17, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M307138200

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