The Functional Interaction between the Paired Domain Transcription Factor Pax8 and Smad3 Is Involved in Transforming Growth Factor-β Repression of the Sodium/Iodide Symporter Gene

Received for publication, July 3, 2003, and in revised form, November 11, 2003
Published, JBC Papers in Press, November 17, 2003, DOI 10.1074/jbc.M307138200

Eugenia Costamagna‡, Bibian García§, and Pilar Santisteban¶
From the Instituto de Investigaciones Biomédicas “Alberto Sols,” Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid. Arturo Duperier 4, 28029 Madrid, Spain

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 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 (acALK5). Using internal deletions of the promoter, we defined 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 down-regulation 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.

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 a key role in thyroid pathophysiology as the route by which iodide reaches the gland for thyroid hormone biosynthesis and as a means for diagnostic scintigraphic imaging and radioiodide 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 thyroid transcription factor-1 (TTF-1)-binding sites that have no effect on NIS transcription, Pax8 binding sites, and a degenerate cAMP responsive element sequence. Full TSH-cAMP-dependent transcription requires Pax8 binding and the integrity of the cAMP responsive element-like sequence in the rat and human NIS promoters (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 differentiation, being the main mediator of thyroid gene 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, and deoxymethasone also play a role in NIS modulation (3, 4, 18–21). Both infiltrating inflammatory cells and thyroid follicular cells themselves produce cytokines, which affect thyroid function and growth and cause immunological changes in the gland. The cytokines studied include transforming growth factor-β (TGF-β).
β), tumor necrosis factor-α and -β (TNF-α and -β), interferon γ (INF-γ), and interleukins (ILs), all of which exert an inhibitory effect on thyroid function, including decreased NIS expression and I⁻ uptake (4, 18, 19, 22).

The TGF-β family of cytokines regulates cell proliferation, survival, adhesion, migration, differentiation, and specification of developmental fate. TGF-β 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-β family members, and they are the only TGF-β-specific receptors with a demonstrated ability to propagate signals (23–25, 28, 29). TGF-β binds to a specific serine/threonine kinase type II receptor on the cell surface, resulting in activation of the dormant kinase activity of the 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), antagonize R-Smad activity. Smads thus signal transiently in the nucleus by controlling the 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 the 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 to –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 down-regulation of NIS gene expression in thyroid follicular cells and might be extended to other genes transactivated by the TGF-β family of receptors with a demonstrated ability to propagate signals (23–25, 28, 29).

**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 a six-hormone mixture (1 nM TSH, 10 μg/ml insulin, 10 ng/ml somatostatin, 5 μg/ml transferrin, 10 μg hydrocortisone, and 10 ng/ml glycy1-L-histidyl-L-tosine acetate; complete medium). The effects of hormones and growth factors was studied by starving near confluent cells for TSH and insulin (23–25, 28, 29).

**Hybridization**—Total RNA was isolated using RNA extraction and Northern blot analysis. Total RNA (20 μg) was separated in 1% agarose gels containing 0.44 M formaldehyde. RNA was blotted onto membranes from PC Cl3 cells were collected in buffer A (10 mM Heps-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) and sedimented at low centrifugal force to pellet nuclei and unbroken cells. The supernatant (cytoplasmic extract) was collected, and the nuclear pellet was resuspended in buffer C (20 mM Heps-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) (38). Cellular debris was removed by centrifugation, and the supernatant fraction was collected. Nuclear, cytoplasmic, and membrane protein concentration were determined according to Bradford (59). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell). Ponceau S staining of a blot fragment is shown.

**Plasmids**—The 2.85-kb 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 described previously (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, and pDNA3-FLAG-Smad7, encoding FLAG or hemagglutinin (HA) epitope-tagged human Smad members, and pCMV5-TßRI (ALK-5), harboring a constitutively activated form of the TGF-ß receptor type I (TßRI), have been described previously (40–42). Full-length Pax8 and the carboxyl-terminal deletion ΔPax8 were subcloned in our laboratory in the pcDNA3.1+ vector.

**Transfection**—PC Cl3 cells were plated at 6 × 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 a LUC and Renilla activity assay using the Dual-Luciferase reporter assay system (Promega). In cotransfection experiments, the amount of DNA was normalized using the corresponding insertless expression vector as the carrier.

**GST Pull-down Analysis**—For GST pull-downs, GST fusion constructs of Smad3 or Pax8 were expressed in Esherichia coli and purified using glutathione-Sepharose 4B beads (Amersham Biosciences). Equal amounts of GST or GST-Smad 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]methionine 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, 0.1% SDS) and 25 μl of wash buffer containing 20 mM Heps-KOH, pH 7.9, 1.5 mM MgCl₂, 250 mM sucrose, 1 mM EDTA, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. Cells homogenates were centrifuged (100,000 × 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 Heps-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) and sedimented at low centrifugal force to pellet nuclei and unbroken cells. The supernatant (cytoplasmic extract) was collected, and the nuclear pellet was resuspended in buffer C (20 mM Heps-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) (38). Cellular debris was removed by centrifugation, and the supernatant fraction was collected. Nuclear, cytoplasmic, and membrane protein concentration were determined according to Bradford (59). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell). Ponceau S staining of a blot fragment is shown.
10% glycerol, and protease inhibitors), and specifically bound proteins were detected by SDS-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 were detected by Western blot analysis. Specific immunodetection was carried out by incubation with anti-Pax8 or anti-Smad3 antibodies (Santa Cruz Biotechnology).

**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 (Fig. 1A) and Western blot (Fig. 1B) 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 (Fig. 1, A and B). The maximum inhibitory effect was reached at 10 ng/ml TGF-β. These data demonstrated that TGF-β interferes with TSH induction of NIS gene expression.

**TGF-β Down-regulates 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). 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).

**Band Shift Assay**—A 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 × 10^6/g), 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 dithiothreitol, 150 ng/ml 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× Tris borate-EDTA. For competition, an excess of cold oligonucleotide was added to the reaction mixture.
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 down-regulates TSH-induced NIS gene transcription, we studied the intracellular mediators of the TGF-β pathway. A signaling cascade is initiated after TGF-β binding, resulting in R-Smad activation, oligomerization with Smad4, and the 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 cotransfected 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 the 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 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 prevented inhibition of the NIS promoter construct by acALK-5 (Fig. 3D), indicating the specificity of Smad7 in blocking Smad-mediated transcriptional repression. Altogether, 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). Because 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, because TSH alone did not induce Smad translocation to the nucleus (compare first and second lanes of Fig. 4, A and 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 the NIS Promoter—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, oligo PB, spanning −2409 to −2377 bp of the rNIS promoter (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 with the control (lane 7). Complex specificity was confirmed by competition with the related (lane 3) but not with 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, because 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 carboxyl-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 and 6) but not with GST beads in a cell-free system (lanes 2 and 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 a 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 (Fig. 6B, lanes 1 and 7) or cells treated with TSH plus TGF-β (Fig. 6B, lanes 2 and 8). Interaction between Smad3 and Pax8 was confirmed through the complexes formed with GST-Smad3 beads and endogenous Pax8 (Fig. 6B, lanes 5 and 6) as well as with GST-Pax8 beads and endogenous Smad3 (Fig. 6B, lanes 11 and 12). No interaction was found with GST beads (Fig. 6B, lanes 3 and 4 and 9 and 10).

**DNA Binding Activity of Pax8 to the NIS Promoter Is Reduced by Smad3**—Here, we demonstrated that TGF-β decreases Pax8 DNA binding to the 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, performed 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 and B, lanes 5–9), the amount of Pax8-DNA complex decreased. No effect was found with GST-alone (Fig. 7, A and B, lanes 2–4). These findings indicate that Smad3 is able to inhibit a Pax8-DNA complex dependent upon the association of Smad3 with the Pax8 paired domain.

**TGF-β Decreases mRNA and Protein Levels of Pax8 in PC Cl3 Cells**—We also studied expression of Pax8 mRNA and

---

**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 TSH alone and then with TSH plus TGF-β for different times (0.25–4 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 α-Sp1 (A) or α-actin (B) antibodies.

**Fig. 5.** TGF-β decreases the binding of Pax8 to its response element in the NIS promoter. Upper panel, schematic representation of the NIS promoter structure. CREL-BF, cAMP-responsive element-like-binding factor. Lower panel, starved PC Cl3 cells were treated for 24 h with TSH plus TGF-β or TSH alone and then with TSH plus TGF-β for different times (0.25–4 h). Cells were then harvested for nuclear extract preparation. A band shift assay was performed with a 32P-labeled oligonucleotide corresponding to the Pax8-binding site (site 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 of the N-terminal paired box DNA-binding (PD), repressor (RD) and activating (AD) domains are indicated. 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 an 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 from PC Cl3 cells treated with TSH (24 h) plus TGF-β (1 h) (lanes 2 and 8) were incubated with GST-coupled 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.

**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, which is also dependent on TGF-β dosage. 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 thyroglobulin, 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 glands (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 TGF-β-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 the thyrocyte membrane (31). We subsequently examined Smad involvement as TGF-β-dependent effectors in NIS transcription. Our studies with Smad3 and Smad4 expression vectors indicated a role for the 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, thereby 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
promoter (21). Results of transient transfection assays confirm transcription, we used the 2.8-kb DNA fragment of the rat Starved PC Cl3 cells were treated for 24 h with TSH plus TGF-
region responsible for TGF-
tional activation in a dose-dependent manner. To delimit the B
A

probe is shown. As a loading control, the membrane was hybridized with an 18 S RNA probe. B, a representative Western blot of nuclear extract (15 μg) was probed with α-Pax8 antibody. As a loading control, the membrane was hybridized with α-Sp1 antibody.

demonstrated not only the presence of Smad3 and Smad4 in PC CI3 cells but also their activation and nuclear accumulation after TGF-β treatment, confirming data in porcine thyroid cells (31). In support of our findings is the fact that Smad3 mediates TGF-β-dependent inhibition of a number of genes (29, 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 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 trans-activation 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. Because 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). Because 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 recruit 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 the NIS promoter, resulting in a functional interaction between these proteins. A similar model of repression has been observed with Pax2/5/8 family members and helix-loop-helix Id proteins, where the interaction between them involves the paired domain of Pax2/5/8 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 down-regulation 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 radiodine 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 CI3 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 down-regulation of the Pax8 expression. Because Pax8 is important for TSH-dependent transcriptional activation, the TGF-β/Smad signal prevents the Pax8-mediated NIS gene transactivation.

We have demonstrated physical and functional interaction between Smad3 and the Pax8 transcription factors. 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.

Acknowledgments—We are indebted to Dr. A. Fusco (Università degli Studi di Napoli, Naples, Italy) for providing PC CI3 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 GST-Pax8 and pcDNA3.1 + ΔPax8 constructs, Dr. R. Di Lauro (Istituto Zoologico, Naples, Italy) for providing Pax8 cDNA and Pax8 antibody, and Dr. M. Carrasco (Albert Einstein College of Medicine, Bronx, NY) for the NIS cDNA and the NIS antibody. We thank Catherine Mark for her linguistic assistance.

REFERENCES

The Functional Interaction between the Paired Domain Transcription Factor Pax8 and Smad3 Is Involved in Transforming Growth Factor-β Repression of the Sodium/Iodide Symporter Gene
Eugenia Costamagna, Bibian García and Pilar Santisteban

doi: 10.1074/jbc.M307138200 originally published online November 17, 2003

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

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 53 references, 17 of which can be accessed free at http://www.jbc.org/content/279/5/3439.full.html#ref-list-1