

# Follicular Thyroglobulin Suppresses Iodide Uptake by Suppressing Expression of the Sodium/Iodide Symporter Gene

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## ABSTRACT

A major function of the thyrocyte is to take up and concentrate iodide. This is needed for thyroid hormone synthesis and is accomplished by the sodium iodide symporter (NIS), whose expression and activity are up-regulated by TSH. Recently, we reported that follicular thyroglobulin (TG) is a potent suppressor of thyroid-specific gene expression and can overcome TSH-increased gene expression. We suggested this might be a negative feedback, autoregulatory mechanism that counterbalanced TSH stimulation of follicular function. In this report, we support this hypothesis by coordinately evaluating TG regulation of NIS gene expression and iodide transport. We show that physiological concentrations of TG similarly and significantly suppress TSH-increased NIS promoter activity, NIS protein, and NIS-dependent iodide uptake as well as RNA levels. We show, *in vivo*, that TG accumulation at the apical membrane of a thyrocyte facing the

follicular lumen is associated with decreased uptake of radioiodide. It is likely, therefore, that TG suppresses NIS-dependent iodide uptake and NIS gene expression *in vivo*, as is the case *in vitro*. RNA levels of NIS and vascular endothelial growth factor/vascular permeability factor, which has been reported to be TSH regulated and possibly associated with TSH-increased iodide uptake, are coordinately decreased by follicular TG as a function of concentration and time. Also, removal of follicular TG from the medium, but not TSH, coordinately returns NIS and vascular endothelial growth factor/vascular permeability factor RNA levels to their TSH-stimulated state. TG accumulated in the follicular lumen appears, therefore, to be a negative feedback regulator of critical TSH-increased follicular functions, iodide uptake, and vascular permeability. (*Endocrinology* **140**: 5422–5430, 1999)

A MAJOR FUNCTION of the thyroid is to take up and concentrate iodide. This function is mediated by the sodium/iodide symporter (NIS), which is expressed on the basal membrane of follicular cells (1). TSH-increased concentrative iodide uptake is associated with TSH-increased NIS RNA levels and gene expression (1, 2).

We have recently shown (3) that thyroglobulin (TG) accumulated in the follicular lumen is a potent suppressor of NIS RNA levels in addition to RNA levels of the thyroid-specific or thyroid-restricted genes, *i.e.* TG, the thyroid peroxidase (TPO), and the TSH receptor (TSHR). This reflects the ability of follicular TG to suppress the expression of thyroid-specific or thyroid-restricted transcription factors that regulate expression of the TG, TPO, TSHR, or NIS genes: thyroid transcription factor-1 (TTF-1), TTF-2, and Pax-8 (3). We showed that the action of TG counterbalanced the action of TSH on several of these genes and speculated that TG might be a negative feedback autoregulator of the function of thyrocytes *in vitro* and *in vivo* (3).

The follicle is the functional unit of the thyroid. However, the function of individual follicles with respect to iodide uptake is not synchronized, but heterogeneous (4–9). Demonstration of TG regulation of iodide uptake *in vivo* and at the

apical membrane requires, therefore, a histochemical approach. Additionally, TSH-regulated iodide uptake by the thyrocyte does not occur as an isolated biological event. The thyroid gland is a hypervascularized tissue, in which a fine capillary network surrounds each thyroid follicle (10). As inorganic iodide is supplied by the blood, the possibility has been considered that there is coordinate TSH regulation of vascular permeability and concentrative iodide transport (11). Exchange of nutrients between the blood stream and the perivascular space surrounding the thyrocyte is regulated by both osmotic pressure (12–14) and specific factors that alter vascular permeability, *i.e.* vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) (15–17). Recent evidence indicates that thyrocytes produce VEGF/VPF and that its synthesis and secretion are regulated by TSH (11, 18, 19). The possibility exists that TSH-enhanced VEGF/VPF alters permeability and enhances the availability of iodide to NIS, thereby facilitating concentrative iodide uptake into the thyrocyte (11). The effect of follicular TG on VEGF/VPF and whether its control is coordinate with the effects of TG on NIS are unknown.

In this report, we focused on demonstrating that the suppressive action of follicular TG on TSH-increased NIS RNA levels was caused by suppression of NIS gene expression at a transcriptional level, as we showed is the case for the TG, TPO, and TSHR genes (3), and, equally importantly, was associated with negative regulation of TSH-increased NIS protein and iodide uptake. These studies were undertaken to test the hypothesis that TG suppression of thyroid-specific or

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thyroid-restricted genes was associated with a negative autoregulatory effect on function *in vitro* and *in vivo* and that TG suppression could, indeed, counterbalance the action of TSH on that function. In the process, we amplify our understanding of the action of TG by showing that TG coordinately suppresses VEGF/VPF and NIS RNA levels in TSH-treated cells in association with decreased iodide uptake. This suggests there may be coordinate control of vascular permeability and NIS to maximize iodide uptake by the thyroid.

## Materials and Methods

### Culture and treatment of cells

Rat FRTL-5 thyroid cells (ATCC CRL8305, American Type Culture Collection, Manassas, VA) were the F<sub>1</sub> subclone provided by the Interthyr Research Foundation (Baltimore, MD). They were grown in Coon's modified F-12 medium (Sigma Chemical Co., St. Louis, MO) containing 5% heat-treated, mycoplasma-free calf serum (Life Technologies, Inc., Grand Island, NY), 1 mM nonessential amino acids (Life Technologies, Inc.), and a supplement of six hormones or growth factors (termed 6H) including bovine TSH ( $1 \times 10^{-10}$  M), insulin (10  $\mu$ g/ml), cortisol (0.4 ng/ml), transferrin (5  $\mu$ g/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml). The cells had the properties previously described (3, 20), were diploid, and between their 5th and 25th passage. Their doubling time with TSH was  $36 \pm 6$  h; without TSH, they did not proliferate. After 6 days in medium with no TSH, addition of  $1 \times 10^{-10}$  M TSH resulted in 10-fold or greater increases in cAMP levels, iodide uptake, and thymidine incorporation into DNA. Fresh medium was added every 2 or 3 days, and cells were passaged every 7–10 days. Bovine thyroglobulin was added directly to the medium at a concentration of 0.1, 1, or 10 mg/ml.

### Iodide uptake measurements

Iodide uptake was measured as previously described (21). Briefly, cells grown in 24-well plates were washed with HBSS containing HEPES buffer, pH 7.4, and were incubated with 0.1  $\mu$ Ci carrier-free  $^{125}$ I at 37 C for 40 min. Cells were washed with ice-cold PBS, pH 7.4, and lysed with ice-cold ethanol by placing them at  $-20$  C for 20 min, at which point the radioactivity of the iodide taken up by the cell was measured.

### Iodide autoradiography and TG immunohistochemistry

Wistar strain male rats were injected ip with 1 mCi  $^{125}$ I 2 h before animals were killed in accord with an approved institutional protocol. Thyroids were obtained and fixed in Bouin's solution, then embedded in paraffin. Sections were cut, and autoradiography or immunohistochemical staining was performed as previously described (5, 22–24).

### RNA isolation and Northern analysis

Total RNA isolation and Northern analysis were performed by a modification of methods previously described (3, 25, 26). RNA was prepared using RNeasy Mini Kits (QIAGEN, Valencia, CA) and minor modifications of the manufacturer's protocol. Cells were cultured in 10-cm dishes, washed with PBS, recovered with 600  $\mu$ l lysis solution, and passed through a QIAshredder (QIAGEN). After 600  $\mu$ l 70% ethanol were added, the mixture was passed through a spin column and washed with 600  $\mu$ l RW1 wash solution and twice with 500  $\mu$ l RPE wash solution. RNA was eluted with 30  $\mu$ l diethylpyrocarbonate-treated water.

RNA samples were electrophoresed on denatured agarose gels and capillary blotted on a nylon membrane using a Turboblotter (Schleicher & Schuell, Inc., Keene, NH). After UV cross-linking, hybridization was performed as follows. Probes were labeled with [ $^{32}$ P]deoxy-CTP using a Ladderman Labeling Kit (PanVera, Madison, WI) in the presence of random primer, deoxy-NTP, and Bca DNA polymerase. After the Nytran membranes ( $11 \times 14$  cm; Schleicher & Schuell, Inc., Keene, NH) were prehybridized with 10 ml QuickHyb Hybridization Solution (Stratagene, La Jolla, CA) for 1 h at 68 C,  $1 \times 10^7$  cpm radiolabeled probe were added after it had been premixed with 100  $\mu$ l sonicated salmon sperm DNA (Stratagene, La Jolla, CA), heated at 94 C for 5 min, then chilled

on ice. After hybridization for 3 h, membranes were washed in  $4 \times$  SSPE with 0.5% SDS for 30 min at 37 C,  $2 \times$  SSPE with 0.1% SDS for 20 min at 65 C, and  $1 \times$  SSPE with 0.1% SDS for 20 min at 65 C. Reprobing was performed after incubating membranes in 50% formamide, 50 mM Tris-Cl (pH 8.0), and 10% SDS for 1–2 h at 65 C.

The VEGF probe was prepared using RT-PCR and the following primers: 5'-ACAGAAGGGGAGCAGAAA-3' and 5'-GAGGTCTAGT-TCCCGAAA-3'. First strand complementary DNA was synthesized using an Advantage RT for PCR Kit (CLONTECH Laboratories, Inc., Palo Alto, CA). PCR reactions were performed as previously described (3, 25) using the Touchdown PCR procedure (27), a GeneAmp 9600 PCR machine (Perkin Elmer Corp., Norwalk, CT), and Pfu DNA polymerase (Stratagene). Probes for TG, NIS, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously (3, 26).

### NIS promoter-luciferase chimeric plasmids

Genomic sequence from  $-420$  to  $-1$  bp of the NIS promoter (28) was amplified by PCR using rat genomic DNA (Promega Corp., Madison, WI) and forward and reverse primers containing an adaptor sequence to facilitate directional cloning. The 5'-primer had a 5'-XhoI restriction site, 5'-ACGCCTCGAGTCCTTATGGAGCCCGGAAG-3'; the 3'-primer had a HindIII restriction site, 5'-GGCTAAGCTTGGAGACAGGT-GACTCGGTG-3'. PCR products were cloned into plasmid PGL3Basic (Promega Corp.), and inserts were sequenced to ensure there was no misincorporation.

### Transient expression analysis

Lipofectamine (Life Technologies, Inc., Gaithersburg, MD) was used to transfect promoter-reporter gene constructs into FRTL-5 cells (3, 26). Briefly, cells were grown in 6-well plastic plates to about 50% confluence and washed with 2 ml warmed (37 C) serum-free culture medium (6H0), and 1 ml of a premade plasmid/Lipofectamine mixture was added. The plasmid/Lipofectamine mixture was made by incubating 1  $\mu$ g plasmid DNA with 10  $\mu$ l Lipofectamine and 200  $\mu$ l 6H0 medium for 30 min at room temperature, then diluting it with 800  $\mu$ l 6H0 medium. Cells were incubated for 4 h at 37 C in a CO<sub>2</sub> incubator before 4 ml complete medium with serum were added. Fresh medium was added after 24 h, with or without the added ligands or reagents noted in individual experiments, and reporter activity was measured 36 h later. To measure luciferase activity, cells were washed and scraped with 1 ml Dulbecco's PBS, then resuspended and lysed with 30  $\mu$ l  $1 \times$  Reporter Lysis Buffer (Promega Corp.) by repetitive pipetting, 30 times, using a micropipette with a yellow tip (200- $\mu$ l capacity). The lysate was incubated for 30 min at room temperature, frozen on a dry ice, thawed, then centrifuged 4 C for 10 min. Twenty microliters of the supernatant were mixed with 100  $\mu$ l luciferase assay reagent (Promega Corp.) and analyzed using a luminometer (Lumat LB9507, Wallac, Inc., Gaithersburg, MD). Two microliters of the supernatant were taken for protein measurements using bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, IL) and the manufacturer's protocol.

### Western blots

Samples were prepared, and blotting was performed as previously described (29, 30). Minor modifications were as follows. Sample buffer containing Tris, SDS, and  $\beta$ -mercaptoethanol were purchased from Novex (San Diego, CA), and the blocking buffer was PBS containing 0.6% Tween-20, 10% milk protein, and 1% crystalline BSA. Anti-NIS was provided by N. Carrasco (Albert Einstein College of Medicine, Bronx, NY); donkey antirabbit IgG was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) as was the mol wt marker kit. Detection used ECL reagents (Amersham Pharmacia Biotech, Arlington Heights, IL).

### Materials

Highly purified bovine TSH was obtained from the hormone distribution program of the NIDDK, NIH (NIDDK-bTSH; 30 U/mg). Bovine follicular 19S TG was prepared by salt extraction and agarose chromatography as previously described (31–33); each was a single 330-kDa component by electrophoresis in reducing gels containing SDS (31–33). When tested, bovine TG from Sigma Chemical Co. gave the same results.

The source of the other materials was Sigma Chemical Co. unless otherwise noted.

Statistical significance

All experiments were repeated at least three times with different batches of cells. Values are the mean ± SD of these experiments where noted. Significance between experimental values was determined by two-way ANOVA; *P* < 0.05 was significant.

Results

Follicular TG suppresses TSH-increased NIS promoter activity and protein levels in addition to RNA levels

FRTL-5 cells maintained in medium without TSH (5H medium) had very low NIS RNA levels, but significant levels of TG RNA (Fig. 1A, lane 1), because insulin and insulin-like growth factor I in the serum are potent positive regulators of TG, not NIS, gene expression (1, 2, 34). The presence of TSH increased NIS RNA levels 10- to 20-fold, but increased TG RNA levels only 2-fold (Fig. 1, A, lane 2, and B), consistent with data in previous reports (1, 2, 34). When 19S follicular TG was added to the medium, TSH-increased NIS and TG RNA levels were markedly reduced (Fig. 1, A, lane 4, and C). In the absence of TSH, 19S follicular TG added to the medium of the cells also decreased basal TG RNA levels (Fig. 1A, lane 3); in the case of NIS, this was small and more difficult to visualize because of the very low NIS RNA levels in the absence of TSH. The effect of follicular TG on TG RNA levels in 5H medium, which contains added insulin and insulin-like growth factor I in the serum, was associated with its action to decrease TTF-2 levels (3).

The concentrations of TG used in these experiments were comparable to physiological concentrations of TG in the colloid measured by fine needle aspiration biopsy, i.e. 0.1–3 mg/ml in normal human thyroids and up to 14 mg/ml in colloid nodules (35). TG did not similarly alter the expression of ubiquitous genes such as GAPDH (Fig. 1, A–C) and β-actin (data not shown). The suppressive effect of a physiological

concentration of follicular TG can, therefore, overcome a maximal effect of TSH, particularly in the case of NIS whose RNA levels were predominantly increased by TSH.

In our previous report (3), we showed that follicular TG suppression of the TG, TPO, and TSHR genes reflected an ability of TG to modulate their expression at a transcriptional level. The same is the case for the TSH-induced expression of the NIS gene. Thus, TSH increased expression of a NIS promoter-luciferase chimera transiently transfected into FRTL-5 cells was decreased by the addition of a physiological concentration of 19S follicular TG to the medium (Fig. 2). The same concentrations of BSA, IgG, and mannitol had no effect (data not shown). TSH and TG had no effect on the activity of the PGL-3Basic vector control. The follicular TG caused a small decrease in basal NIS promoter activity consistent with its small effect on basal NIS RNA (Fig. 1, lane 3).

Coincident with the ability of follicular TG to decrease TSH-increased NIS promoter activity and RNA levels, follicular TG decreased TSH-increased NIS protein levels when measured as a function of time and TG concentration (Fig. 3).

Follicular TG suppresses TSH-increased iodide uptake both in vitro and in vivo

The ability of follicular TG to decrease TSH-increased NIS RNA and protein levels as well as NIS promoter activity was associated with the ability of follicular TG to decrease TSH-increased iodide transport (Fig. 4). Thus, when iodide uptake in FRTL-5 cells cultured with TSH was evaluated, TG significantly suppressed uptake as a function of time (Fig. 4A) and TG concentration (Fig. 4B). The same concentrations of BSA had no such effect (Fig. 4, A and B), nor did the same concentrations of IgG or mannitol (data not shown). The effect of 19S follicular TG on iodide transport was similar to its effect on NIS RNA levels as a function of concentration and time, as shown below (Figs. 6 and 7). However, the effect was less than expected based on the effect of the TG on NIS

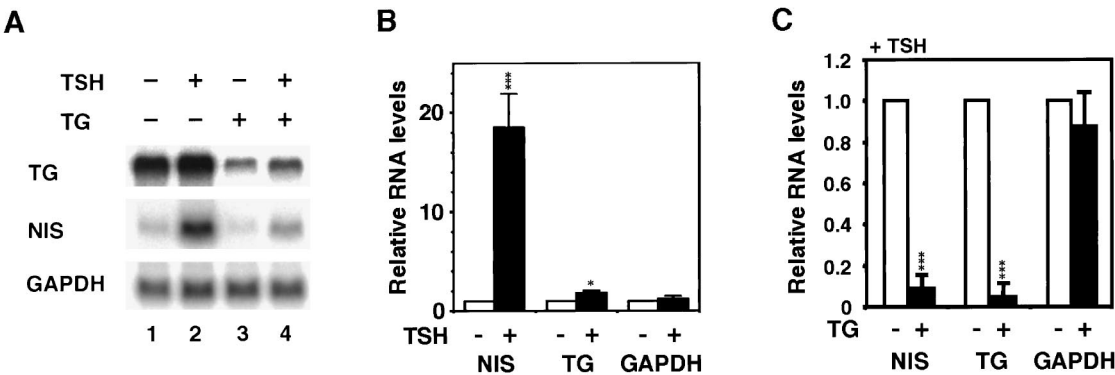


FIG. 1. The suppressive effect of a physiological concentration of follicular TG on NIS RNA levels can overcome the stimulatory effect of TSH. FRTL-5 cells were grown in TSH-containing 6H medium until 60% confluence and then cultured without TSH for 6 days. As FRTL-5 cells maintained multiple days in TSH are not synchronized, a significant fraction of cells complete the cell cycle already initiated when TSH is withdrawn. Thus, the cells are 80–90% confluent by the end of the TSH withdrawal period and the start of the experiment. Bovine TSH at a final concentration of  $1 \times 10^{-10}$  M, which can maximally increase NIS RNA levels in our experiments (data not shown), and/or 19S bovine follicular TG at a final concentration of 10 mg/ml were added to the medium. After cells were cultured for 48 h, total RNA was purified and sequentially hybridized with the noted radiolabeled probes (A). Densitometric analysis of Northern blots were performed, and changes in RNA levels caused by the addition of TSH (B) or by the suppressive effects of follicular TG (C) were calculated. In B, RNA levels in the absence of TSH were set as unity for each gene to emphasize the relative TSH effect. In C, the RNA level in the absence of added follicular TG was set at unity for each gene to portray the magnitude of the effect of the added TG on the original RNA levels of NIS and TG. A represents a typical result of a single experiment; B and C depict the mean ± SD of three different experiments on different batches of cells.



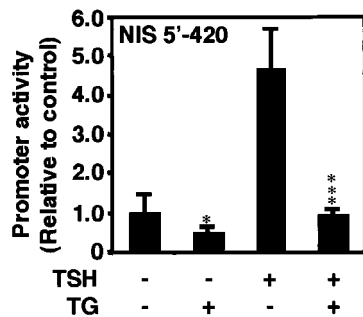


FIG. 2. Ability of exogenous follicular TG to decrease TSH-increased NIS promoter activity in rat FRTL-5 thyroid cells, as assessed by transient expression analysis. FRTL-5 cells were transfected as described (see *Materials and Methods*), and fresh medium containing no TSH, *i.e.* 5H medium with 5% calf serum (5H5), was added after 24 h. To duplicate cultures, fresh medium that had additionally contained  $1 \times 10^{-10}$  M TSH,  $1 \times 10^{-10}$  M TSH plus 1 mg/ml TG, or 1 mg/ml TG alone were added. Reporter activity was measured 36 h later and normalized for transfection efficiency, which was not affected by the presence of TG subsequently added to the medium. Values are expressed relative to values measured using the PGL-3Basic promoter alone, *i.e.* with no NIS insert, whose activity was set at 1. Data are the mean  $\pm$  SD of three different experiments performed in triplicate. Three asterisks represent a significant TG-induced decrease at  $P < 0.001$ .

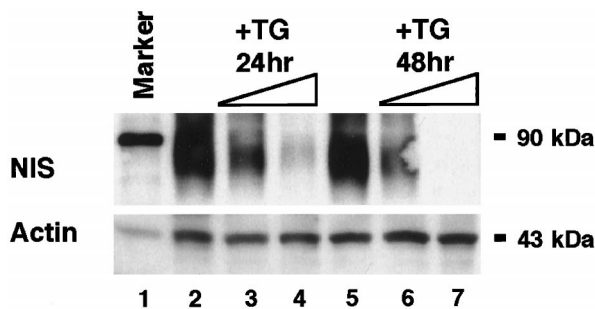


FIG. 3. Follicular TG suppresses NIS protein levels measured in Western blots. FRTL-5 cells maintained in the presence of TSH were treated with 1 mg/ml 19S Agarose-purified, salt-extracted 19S follicular TG from fresh glands (lanes 3 and 6) or 10 mg/ml TG (lanes 4 and 7) for 24 and 48 h, as noted. Control cells with no added TG are shown in lanes 2 and 5. NIS protein was measured by Western blotting, as was actin (see *Materials and Methods*). Similar results were obtained in three different experiments using different batches of cells.

protein. At 24 and 48 h, using 10 mg/ml follicular TG, levels of this NIS protein were about 10% of control levels and negligible, respectively (Fig. 3). Iodide uptakes at 24 and 48 h, in contrast, were about 30% and 10% of control values using 10 mg/ml follicular TG (Fig. 4, A and B). Using 1 mg/ml TG, NIS protein was about 25% of control levels after 48 h, whereas iodide uptake was significantly higher, about 50% of control values.

Residual iodide uptake despite very low NIS protein levels may reflect several possibilities. One is the existence of other iodide-transporting systems or channels in FRTL-5 cells, *i.e.* an apical membrane porter or other chloride/iodide channels, both of which have been described previously (36, 37). A second possibility is that there may be active and inactive forms of NIS protein that are regulated by posttranslational modifications, changes in subcellular localization, or protein half-life. This possibility was raised in studies of TSH-reg-

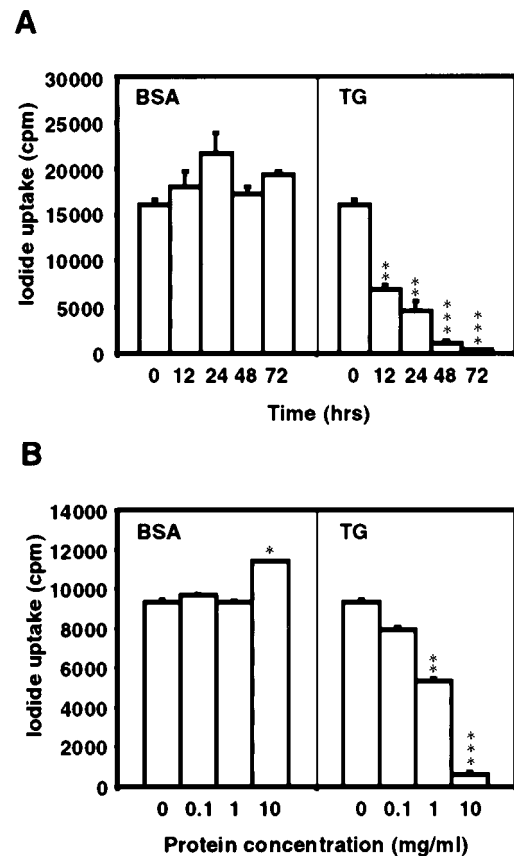


FIG. 4. Follicular TG suppresses uptake of  $^{125}\text{I}$  in FRTL-5 cells as a function of time (A) and concentration (B). In A and B, FRTL-5 cells maintained in the presence of TSH were treated with 19S follicular TG, and iodide uptake was measured as described in *Materials and Methods*. Results are the mean  $\pm$  SD of triplicate assays from three different experiments. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . In A, the TG concentration was 10 mg/ml; in B, the time was 48 h.

ulated NIS protein *vs.* activity by Kogai *et al.* (2) and the Carrasco group (38), who also noted discrepancies in the two parameters. This phenomenon is further examined in the *Discussion*.

To determine whether follicular TG down-regulated iodide uptake *in vivo*, uptake of iodide in individual cells was studied together with follicular TG binding to the apical membrane. A combined autoradiographic/histochemistry approach was used. In previous reports, TG suppression of thyroid-specific genes appeared to be associated with TG moieties that bound to an apical membrane receptor (3) and was associated *in vivo* with TG bound to the rim of the follicular lumen (39). To determine whether TG that was bound to the apical membrane might be associated with suppressed iodide uptake in thyrocytes surrounding the lumen of a follicle, Wistar rats were injected with  $^{125}\text{I}$ , and thyroid tissue sections were immunostained with anti-TG antibody after autoradiography to visualize the TG bound to the apical membrane in relation to the radioiodine incorporated into the thyrocytes. There was a clear inverse correlation between follicular TG accumulation on the apical surface of the cells and iodine uptake in cells surrounding the follicular lumen (Fig. 5).

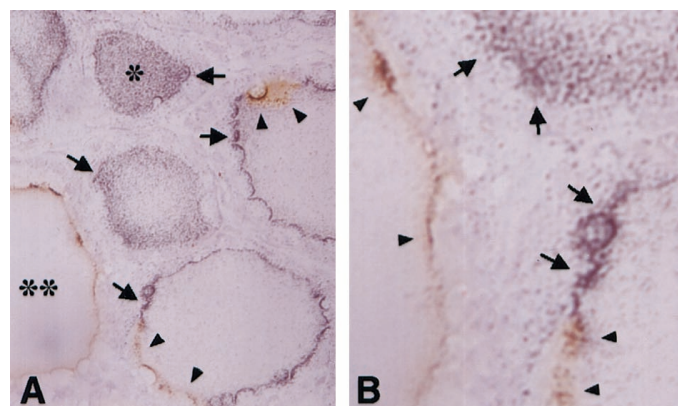


FIG. 5. Inverse correlation of follicular TG accumulation on the apical membranes of cells and iodide uptake by cells *in vivo*. Rats were injected ip with  $^{125}\text{I}$  and killed 2 h later. Thyroids were fixed in Bouin's solution under conditions that removed the colloid from the follicular lumen and then embedded in paraffin. Sections were cut, and radioautography and immunohistochemical staining were performed. Follicular TG was visualized as a brown coloration by immunoperoxidase staining, and uptake of  $^{125}\text{I}$  was visualized by autoradiography. In A, a follicle with a high level of accumulated iodide is indicated by an asterisk, and a follicle in which iodide uptake is poor is indicated by two asterisks. Magnification,  $\times 40$ . Arrows indicate the accumulation of silver grains, and arrowheads indicate apical TG immunostaining. The follicle with poor iodinated uptake (\*\*) has a rim of brown immunostaining reflecting a rim of TG uniformly bound on the apical border of the cells. There is poor iodide uptake by the cells surrounding the follicular lumen in this follicle. In B, at higher magnification ( $\times 200$ ) the heterogeneity of the phenomenon is also clear. There are areas within a follicle where suppressed uptake is evident where TG is bound (brown immunostaining). There are also areas with higher numbers of cells taking up iodide (arrows) and where TG bound to the apical membrane, as shown by immunohistochemical staining (arrowheads), is nil.

Thus, in Fig. 5, TG immunostaining was visible as brown coloration, and  $^{125}\text{I}$  was visualized as dark silver grains detected by autoradiography. Consistent with heterogeneity of follicles (5, 22), some follicles showed dense radioiodine within cells surrounding the follicular lumen (Fig. 5A, follicle with single asterisk and arrows), but others showed almost no uptake (Fig. 5A, follicle with double asterisk). Iodide uptake measured by the dark silver grains was not associated with TG immunostaining (brown color) at the apical border of the cells surrounding the follicular lumen, whereas TG immunostaining was not associated with iodide uptake in adjacent thyrocytes. In general, a follicle that had dense iodide uptake in the cells had poor TG immunostaining (Fig. 5A, asterisk), whereas a follicle with no iodide uptake in the cells had a rim of TG immunostaining along the apical membrane (Fig. 5A, double asterisk). Higher magnification indicates that the TG exists on the surface of the cells facing the follicular lumen rather than within the cytoplasm of the follicular epithelium (Fig. 5B), consistent with evidence that there is a rim of poorly iodinated TG bound to the apical membrane (31–33, 39). The high power magnification shows more clearly that even within the same follicle, localization of radioiodine within a cell and TG accumulation on the apical membrane exhibit an inverse correlation (Fig. 5, A and B, lower right follicle). These results are consistent with our *in vitro* observations shown in Figs. 1–4 and are consistent with our hypothesis (3, 39) that follicular TG regulation of thyroid-specific genes, by regu-

lating levels of thyroid-specific or thyroid-restricted transcription factors, will result in altered thyrocyte function.

#### *Follicular TG coordinately suppresses TSH-increased NIS and VEGF/VPF RNA levels*

One possibility that has been considered, based on previous VEGF/VPF studies (11), is that TSH-increased VEGF/VPF RNA levels might reflect the effects of TSH to increase the availability of iodide to the NIS gene by increasing vascular permeability. If so, there might be coordinate control of NIS and VEGF/VPF RNA levels.

When 19S follicular TG was added to the medium, it caused a simultaneous decrease in TSH-increased RNA levels of NIS and VEGF/VPF as a function of the TG concentration (Fig. 6). TG did not similarly alter the expression of ubiquitous genes such as GAPDH (Figs. 1, 6, and 7) or  $\beta$ -actin (data not shown). Adding the same concentrations of BSA, IgG, or mannitol had no effect on the expression of VEGF/VPF or NIS RNA levels (Fig. 6).

Suppression of TSH-increased NIS and VEGF/VPF RNA levels was maximal by 24 h, whereas suppression of TG RNA levels was maximal at later time periods (Fig. 7, A and B). Moreover, withdrawal of TG from the medium had a similar ability to reverse the suppression of NIS and VEGF/VPF

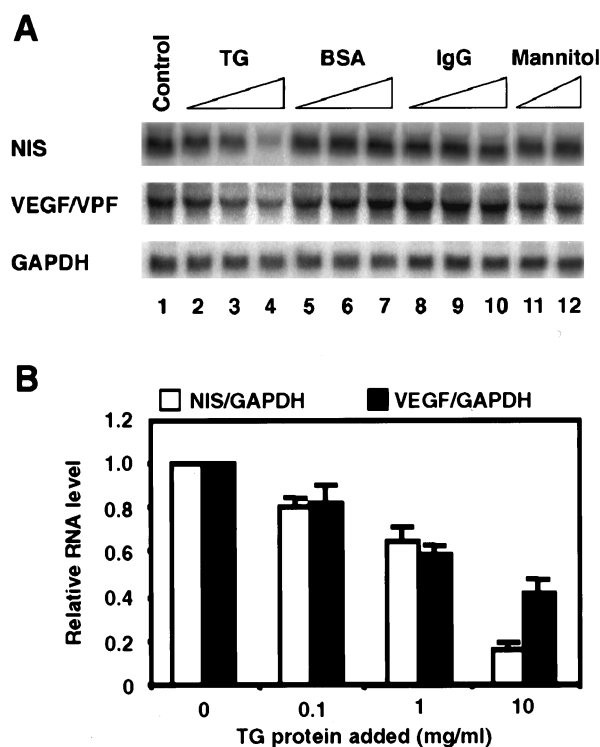


FIG. 6. Follicular TG suppresses TSH-increased levels of NIS and VEGF/VPF mRNA in a concentration-dependent manner. FRTL-5 cells maintained with TSH and 5% serum at 60% confluence were treated with bovine TG, BSA, or bovine IgG at concentrations of 0.1, 1, and 10 mg/ml or mannitol at concentrations of 0.1 and 1 mg/ml. Total RNA was isolated 48 h after treatment, and Northern analysis was performed as described in *Materials and Methods*. A shows a representative Northern blot; B presents the mean values of TG inhibition of TSH-increased NIS or VEGF/VPF mRNA levels  $\pm$  SD as determined after densitometric analysis of assays from three different experiments.

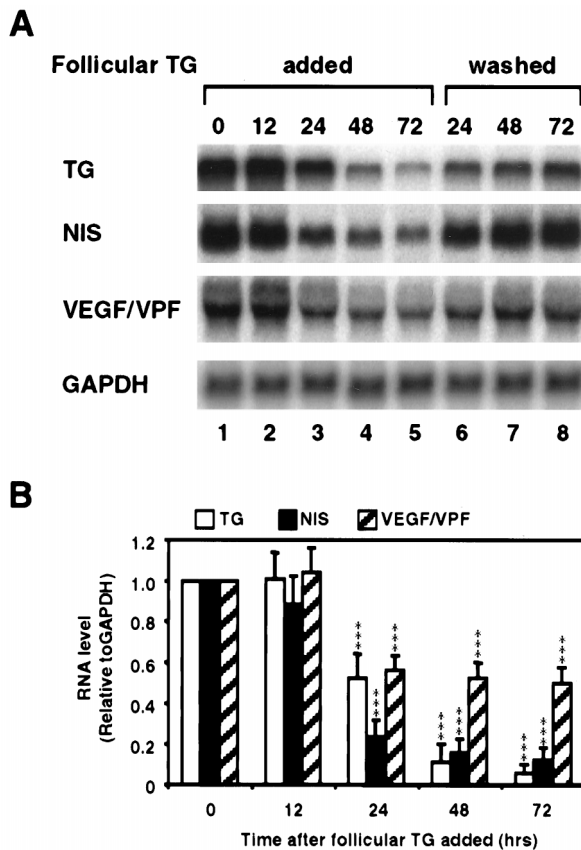


FIG. 7. Addition and removal of TG coordinately suppress and reinitiate, respectively, TSH-increased NIS and VEGF/VPF RNA levels. FRTL-5 cells were treated with 10 mg/ml TG for 12–72 h or were first treated for 12 h, then TG was washed and replaced with normal medium and kept for 24–72 h until total cellular RNA was recovered. Northern analysis was performed as described in *Materials and Methods*. A shows a representative Northern blot; B presents the mean values of TG inhibition of TSH-increased NIS or VEGF/VPF mRNA levels  $\pm$  SD as determined after densitometric analysis of assays from three different experiments.

RNA levels as a function of time (Fig. 7A). Thus, the suppression of TSH-increased NIS and VEGF/VPF messenger RNA (mRNA) levels was TG protein specific and not caused just by protein concentration or osmotic pressure.

### Discussion

TSH supplied from the bloodstream is considered the primary regulator of thyroid function. Recently, however, we described another potential intrinsic regulator of thyroid function, follicular TG. We suggested that TG accumulated in the follicular lumen could be a negative feedback autoregulator of follicular function and counterbalance the action of TSH. This view was based on our previous studies (3, 39), which showed that physiological concentrations of 19S follicular TG could suppress TG, TPO, TSHR, and NIS RNA levels and that this reflected a specific effect of TG on the thyroid transcription factors TTF-1, TTF-2, and Pax-8. With the exception of the TSHR, the effect of TG on TG, TPO, and NIS RNA levels is opposite the effect of TSH. TSH increases TG, TPO, and NIS expression and activity in part at least because of its ability to enhance Pax-8 binding to these genes

(40); follicular TG decreases TG, TPO, and NIS expression and activity because it decreases Pax-8 RNA and protein levels (3). In the case of the TSHR, the effect of TG to decrease TSHR expression and activity (3) is additive with TSH, because the TSHR is regulated only by TTF-1 (40) and because TG as well as TSH decrease TTF-1 RNA levels (39).

The present study was aimed at linking these observations to function, *i.e.* we wanted to show that follicular TG was indeed a negative feedback autoregulator of follicular function and could counterbalance the action of TSH. Additionally, we wanted to amplify our studies of the effect of TG on the NIS gene. Thus, in the previous reports (3, 39), we showed that exogenous 19S follicular TG decreased transcriptional expression of the TG, TPO, and TSHR genes, but we did not demonstrate this for the NIS gene. Additionally, we had not provided evidence for an effect on NIS protein or NIS function, *i.e.* iodide transport.

In this report, we show that 19S follicular TG, at physiological concentrations, *i.e.* at concentrations known to exist in the follicular lumen based on needle biopsies (35), decreases TSH-increased NIS promoter activity as well as RNA levels in rat thyroid FRTL-5 cells and that this is concurrent with decreases in TSH-increased NIS protein and TSH-increased iodide transport. We show that this mechanism, which is defined in rat FRTL-5 cells, has an *in vivo* counterpart. Thus, TG bound to the apical membrane of follicular thyrocytes *in vivo* is associated with suppressed iodide uptake within adjacent thyrocytes when animals are injected with radioiodine. In sum, we show that follicular TG is a counterregulator of TSH-induced NIS function as well as gene expression in a thyrocyte culture system that has been used to characterize the porter (1, 2) and that this likely to be the case *in vivo*.

Studies of TSH-increased NIS RNA levels, protein, and iodide transport have noted discrepancies between TSH-induced changes in NIS protein *vs.* RNA levels and iodide transport (1, 2, 38). They accordingly raised questions about protein half-life and TSH-regulated posttranscriptional regulation of NIS protein (1, 2, 38). The present studies reveal another discrepancy; TG decreases NIS protein levels more than it does iodide transport. This may reflect several possibilities. One is the existence of other iodide-transporting systems or channels in FRTL-5 cells, *i.e.* an apical membrane porter or other chloride/iodide channels, both of which have been described previously (36, 37). These may not be similarly down-regulated by TG. In a recent report we showed that TG increased the RNA levels of pendrin, the presumptive apical membrane iodide porter (41), *i.e.* TG may indeed have opposite effects on iodide porters other than NIS in thyrocytes. A second possibility is that there may be active and inactive forms of NIS protein, which are regulated by posttranslational modifications, changes in subcellular localization, or protein half-life (2, 38). Recent studies of the Carrasco group (38) showed that TSH regulated the subcellular localization and phosphorylation of NIS and raised the possibility this could control differences in the effect of TSH on RNA *vs.* protein levels, protein *vs.* iodide transport levels, and protein stability. A posttranslational modification induced by TSH, *i.e.* phosphorylation, could, for example, alter half-life (2, 38). We have recently shown that phosphorylation can also regulate TG suppression (42, 43). In short, the



more profound effect of TG on NIS protein, than on iodide uptake, emphasizes 1) the still unresolved complexity of iodide transport in thyrocytes despite the discovery of NIS and 2) the complexity of NIS regulation already evident in other studies (2, 38). The present observations may help in future studies of the regulation of NIS protein *vs.* function.

NIS is a transmembrane protein located on the basal membrane of the thyrocyte (1). The TSHR is on the basal membrane, whereas TG appears to operate from the apical membrane, as evidenced by the *in vivo* results in Fig. 5 and suggested in separate reports (31–33, 39, 41–45a). Thus, in a study of propylthiouracil-treated rats (39), we showed that a rim of TG bound to the apical membrane of the follicle *in vivo* was associated with the suppression of TTF-1 mRNA and TG bioynthesis. The existence of a protein on the apical membrane that binds TG that has been vectorially transported to the follicular lumen has been suggested (31–33, 39, 44, 45). A suggested function of the binding protein on the apical membrane is to anchor TG during its reiterative iodination and sialylation processes by TPO and sialotransferase, respectively (31–33, 45). This is consistent with the fact that TG preparations bound to the apical membrane are poorly iodinated, poorly sialylated, and have the highest binding activity to the apical membrane (3, 31–33, 39, 45). This is also consistent with the observation that follicular TG preparations from colloid nodules or iodine deficiency goiters are stronger suppressors than TG from normal thyroid (3). Thus, these data (Fig. 5) (3, 31–33, 39, 44, 45) suggest that TG bound to the apical membrane is an important TG moiety initiating the suppression. However, which receptor binds the TG and how binding of TG to this apical membrane receptor initiates transcriptional suppression of gene expression were unexplained until recently.

One protein that binds TG and was associated with the apical membrane based 1) on its role to vectorially transport TG to the follicular lumen and 2) its involvement in selective fluid endocytosis (45) had properties of the asialoglycoprotein receptor (ASGPR) on liver membranes (31–33). In recent reports (42, 43, 45a), we have unequivocally identified the protein that binds TG as the ASGPR, shown that the ASGPR is on the apical membrane, and shown that TG binding to the ASGPR initiates suppression. Localization of the ASGPR on the apical membrane was evidenced in polarized FRT cells with a specific antibody to the recombinant RHL-1 subunit of the rat thyroid ASGPR (45a). Separate studies (42, 43) showed that the same antibody to the recombinant RHL-1 subunit of the rat thyroid ASGPR prevented the ability of TG to act as a suppressor (42, 43). In summary, the data accumulated in these reports indicates that TG bound to the ASGPR on the apical membrane is the important TG moiety initiating suppression.

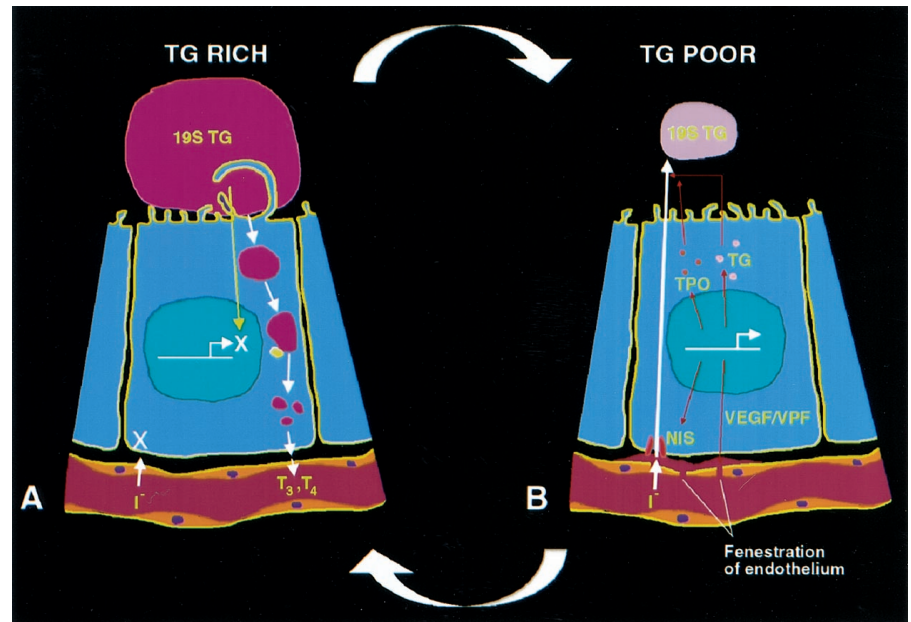
In the case of both TSH and follicular TG the effect on NIS protein levels and function is mediated by transcriptional control of NIS gene expression (Refs. 1–3, 42, 43, 46, and 47 and this report). In the case of TSH, transcriptional regulation mediated by the cAMP signal transduction system seems clear (1, 2, 46, 47). In the case of follicular TG, the exact mechanism by which a large extracellularly stored molecule could physiologically exert its regulatory action at a transcriptional level was unclear. The TG interaction with the

ASGPR on the cell surface in liver or thyroid cells is not known to activate a signal transducing pathway (31–33, 43). Nevertheless, TG has been reported to have phosphorylated tyrosine, serine, and threonine residues (48) and to have a kinase A-like activity (49). In recent reports (42, 43), we have shown that okadaic acid, which inhibits serine/threonine dephosphorylation in cells, eliminates the TG-suppressive activity. We also showed (42, 43) that phosphorylating the serine or serine/threonine residues of TG itself, by autophosphorylation or by phosphorylation with protein kinase A (PKA), respectively, also eliminates TG suppression, but, unlike okadaic acid, enhances transcript levels of the thyroid-restricted genes 2-fold in the absence of a change in TG binding to the ASGPR. We concluded (42, 43) that follicular TG suppression of thyroid-restricted genes is mediated by the ASGPR on the thyrocyte apical membrane and is regulated by a signal system in which phosphorylation of serine/threonine residues on the bound ligand, TG, and possibly on the ASGPR, is an important component. These data provide a hitherto completely unsuspected role for the ASGPR in transcriptional signaling as well as endocytosis and establish a functional role for phosphorylated serine/threonine residues on the TG molecule. Whether it is ASGPR-mediated signaling or endocytosis of a TG fragment that mediates transcriptional suppression is unclear. This may be a phosphorylated fragment, one that is not fully glycosylated, and/or one that is poorly iodinated. These possibilities are under investigation.

The thyroid gland, like other endocrine organs, is a hypervascularized tissue in which a fine capillary network surrounds the follicles (10). In the thyroid, increased VEGF/VPF RNA levels are associated with the hypervascularity of Graves' disease and cystic accumulations of fluid (11, 19). In addition, a recent study has shown that VEGF/VPF can induce fenestrations in the endothelium (17). In the present study, we show that physiological concentrations of follicular TG coordinately regulate VEGF/VPF and NIS RNA levels. This raises the possibility that follicular TG not only suppresses TSH-increased follicular cell function (NIS) directly, but also may alter vascular permeability by secreting VEGF/VPF in paracrine manner. This raises the possibility that TSH and TG coordinately regulate NIS and VEGF/VPF to efficiently improve or inhibit iodide transport into the thyroid follicle and is consistent with the fact that iodide uptake in a whole thyroid correlates with serum iodide levels.

Based on the data herein and those from related reports (3, 39, 42–44, 50–52), we propose a model for the autoregulatory function of accumulated follicular TG on thyroid iodide uptake, thyroid hormone synthesis, and thyroid hormone secretion in follicular thyrocytes surrounding each follicle. In a follicle rich with colloidal TG, the accumulated follicular TG may suppress NIS and VEGF/VPF gene expression, thereby, minimizing iodide uptake (Fig. 8A). At the same time, the transcriptionally directed synthesis of TG and TPO are suppressed (Fig. 8A). In that situation, TSH may act predominantly to cause resorption and degradation of follicular TG and the secretion of thyroid hormones into the bloodstream (Fig. 8A). Because the rate of resorption and degradation of TG exceeds new synthesis and replacement

FIG. 8. Schematic representation of a proposed model of regulation of follicular function by follicular thyroglobulin. See Discussion for details.



of TG in the colloid (50–52), the TG concentration in the lumen of such a follicle will decrease. As the TG content in the follicular lumen decreases (Fig. 8B), transcriptional suppression would be released, and gene expression of NIS and VEGF/VPF would be reinitiated, as would TG and TPO gene expression (Fig. 8B). The synthesized and secreted VEGF/VPF would induce fenestration of the vascular endothelium; this would increase vascular permeability and allow NIS, whose expression is also increased, to take up iodide most efficiently (Fig. 8B). When accumulation of follicular TG reaches a certain level, TG suppression of gene expression would again dominate TSH-stimulated gene expression, and the whole process would be repeated.

This model is consistent with and relevant to follicular heterogeneity (4–9), *i.e.* TG regulation of TSHR gene expression might be a critical transcriptional event determining the sensitivity of an individual follicle to serum TSH, despite a constant TSH supply to all follicles (53–56). Thus, an unknown regulatory mechanism and factor controlling follicular function, independent of TSH, was hypothesized to explain follicular heterogeneity, but was not identified (5, 22, 52). The autoregulatory action of TG and this model would be consistent with the hypothetical factor suggested to account for follicular heterogeneity (5, 22, 52).

In summary, we demonstrate negative feedback regulation of follicular function by follicular TG, iodide transport in particular. We demonstrate that the TG effect on function is independent of TSH, but is mechanistically coordinated with TSH action. We show that TG counterregulates the effect of TSH on thyroid function and is operative at the level of transcription (3).

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