THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 274, No. 35, Issue of August 27, pp. 25099–25107, 1999
Printed in U.S.A.

Follicular Thyroglobulin (TG) Suppression of Thyroid-restricted Genes Involves the Apical Membrane Asialoglycoprotein Receptor and TG Phosphorylation*

(Received for publication, April 22, 1999, and in revised form, June 11, 1999)

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Follicular thyroglobulin (TG) decreases expression of the thyroid-restricted transcription factors, thyroid transcription factor (TTF)-1, TTF-2, and Pax-8, thereby suppressing expression of the sodium iodide symporter, thyroid peroxidase, TG, and thyrotropin receptor genes (Suzuki, K., Lavaroni, S., Mori, A., Ohta, M., Saito, J., Pietrarelli, M., Singer, D. S., Kimura, S., Katoh, R., Kawaoi, A., and Kohn, L. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 95, 8251–8256). The ability of highly purified 27, 19, or 12 S follicular TG to suppress thyroid-restricted gene expression correlates with their ability to bind to FRTL-5 thyrocytes and is inhibited by a specific antibody to the thyroid apical membrane asialoglycoprotein receptor (ASGPR), which is related to the ASGPR of liver cells. Phosphorylating serine/threonine residues of TG, by autophosphorylation or protein kinase A, eliminates TG suppression and enhances transcript levels of the thyroid-restricted genes 2-fold in the absence of a change in TG binding to the ASGPR. Follicular TG suppression of thyroid-restricted genes is thus mediated by the ASPGR on the thyrocyte apical membrane and regulated by a signal system wherein phosphorylation of serine/threonine residues on the bound ligand is an important component. These data provide a hitherto unsuspected role for the ASGPR in transcriptional signaling, aside from its role in endocytosis. They establish a functional role for phosphorylated serine/threonine residues on the TG molecule.

Thyrotropin (TSH),[1] in concert with insulin and insulin-like growth factor-1 (IGF-1), regulates thyroid function (1–3). TSH increases expression of the sodium iodide symporter (NIS), thyroglobulin (TG), and thyroid peroxidase (TPO) genes; this increases concentrative iodide uptake, TG synthesis, and thyroid hormone formation (1–4). NIS, TG, and TPO expression are controlled by thyroid-restricted transcription factors: thyroid transcription factor (TTF)-1, TTF-2, and Pax-8 (5–12). TTF-2 is regulated by insulin/IGF-1 (9, 10), TTF-1 and Pax-8 by TSH/cAMP (13–16).

We have recently shown that TG accumulated in the follicular lumen acts as a feedback suppressor of hormonally-increased thyroid function (17–19). Thus, follicular TG selectively suppresses expression of TTF-1, TTF-2, and Pax-8 (17, 18), thereby altering expression of the TG, TPO, NIS, and TSHR genes, and counter regulating TSH- and insulin/IGF-1-induced changes in these genes (17–19). The follicular TG acts transcriptionally; its suppressive effect is not duplicated by thyroid hormones or iodide (17–19). The mechanism by which follicular TG can act as a transcriptional suppressor is unknown.

TG is synthesized as a 12 S molecule (330 kDa), but forms a 19 S dimer and 27 S tetramer; all three exist in the follicular lumen (20, 21). It has been suggested that newly synthesized TG attaches to a specific binding protein related to the lectin-like asialoglycoprotein receptor (ASPGR) of the liver (22–26)[2] and that the thyroid ASPGR vectorially transports newly synthesized TG to the follicular lumen (22–25). During this vectorial transport process, TG undergoes posttranslational modifications, including phosphorylation, (21, 25, 28–32). At the apical membrane, a membrane-bound sialotransferase and TPO are suggested to reiteratively sialylate and iodinate the TG, allowing its release from the ASGPR into the follicular lumen (22–25). The ASGPR may also be indirectly involved in selective degradation of highly iodinated 19 S TG from the follicular lumen by a process termed "selective fluid pinocytosis" (22, 25, 33). Thus, ASGPR binding of TG at the apical membrane is hypothesized to bind more recently synthesized, poorly iodinated, and poorly sialylated TG, sequestering it, and making it unavailable for fluid pinocytosis. As a result, highly iodinated and sialylated TG molecules, which are free in the follicular lumen, preferentially undergo fluid pinocytosis (22, 25, 33). TG bound to the ASGPR may, however, be internalized with receptor recycling (22, 25, 33).[2] This is suggested to be the basis of the "last come-first served" concept, wherein the most recently synthesized TG attached to the receptor is the first to be degraded (20, 22, 25, 33). ASGPR phosphorylation of serine/threonine residues has been related to ASGPR recycling in the liver, but not to endocytosis (34–36).

In this report we show that the ability of different polymeric forms of TG to suppress thyroid-restricted gene expression is

* This work was supported in part by MURST-Consiglio Nazionale delle Ricerche Biotechnology Program L.95/95. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TSH, thyrotropin; TG, thyroglobulin; TTF, thyroid transcription factor; TSHR, thyrotropin receptor; TPO, thyroid peroxidase; NIS, sodium iodide symporter; BSA, bovine serum albumin; HPGPC, high pressure gel permeation chromatography; IGF-1, insulin-like growth factor-1; MHC, major histocompatibility complex; TBS-T, Tris-buffered saline with Tween 20; PKA, protein kinase A; CAT, chloramphenicol acetyltransferase.

[2] F. Pacifico, D. Liguoro, L. Ulianich, N. Montuori, G. Cali, L. Nitsch, L. D. Kohn, S. Formisano, and E. Consiglio, submitted for publication.

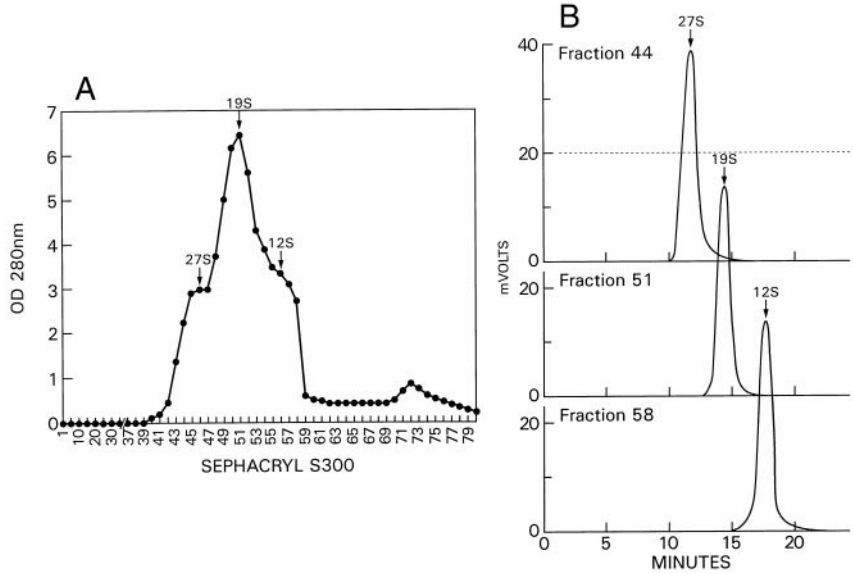

FIG. 1. **Isolation of 27, 19, and 12 S TG moieties by Sephacryl (*A*) and HPGPC chromatography (*B*).** In *A*, 500 mg of salt extracted bovine follicular TG was chromatographed at 4 °C on a 5 × 150-cm Sephacryl S300 column in 0.1 M potassium phosphate, pH 7.5; fractions of 15 ml were collected (21–23). Identification of the 27, 19, and 12 S TG forms was made using calibrated blue dextran markers, myoglobin, and BSA standards; size was confirmed by ultracentrifugation (21–23). In *B*, 27, 19, and 12 S TG forms from individual fractions obtained using the Sephacryl column were rechromatographed by HPGPC (see "Materials and Methods"). Identification of the different TG forms was confirmed by ultracentrifugation (21–23).

related to their ability to bind to the ASGPR, which has been separately located on the apical membrane of thyrocytes.[2] In addition, we show that autophosphorylation of TG, which has been shown to be restricted to phosphoserine residues (37), not only eliminates the ability of TG to be a transcriptional suppressor of thyroid-restricted genes, it also allows the TG to act as an enhancer of their expression. Treatment of cells with an inhibitor of serine/threonine phosphatase activity, okadaic acid, also eliminates TG suppression. We suggest that follicular TG acts as a regulator of thyroid-restricted gene expression by binding to the ASGPR on the apical thyrocyte membrane and that phosphorylation of TG regulates the suppressive effect. The data are the first to describe a role for the ASGPR in transcriptional signaling and a biologic role for phosphorylated TG, particularly its phosphoserine residues.

MATERIALS AND METHODS

Cell Culture—The F1 subclone of FRTL-5 rat thyroid cells (Interthyr Research Foundation, Baltimore, MD) (38, 39) was grown in Coon's modified F-12 medium containing 5% calf serum (Life Technologies, Inc.), 1 mM nonessential amino acids (Life Technologies, Inc.), and a mixture of six hormones (6H) including bovine TSH (1×10^{-10} M) (38, 39). The FRTL-5 cells were diploid, between their 5th and 25th passage, and had the properties previously described (17–19, 38, 39). Their doubling time with TSH was 36 ± 6 h; they did not proliferate without TSH; and, after 6 days in medium with no TSH, addition of 1×10^{-10} M TSH resulted in 10-fold or better 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. In some experiments, cells were changed to 5H medium containing no TSH for a period of 6 days before experiments were initiated.

Thyroglobulin Preparations—Bovine follicular TG was prepared by salt extraction of sliced, fresh thyroid glands, ammonium sulfate precipitation, and gel filtration chromatography on Sephacryl S-300 (Amersham Pharmacia Biotech, Uppsala, Sweden) in 0.1 M potassium phosphate, pH 7.4 (22–24, 31). Procedures were carried out at 0–4 °C in the presence of protease inhibitors (Complete; Roche Molecular Biochemicals) and 150 μM phenyl phosphate (Sigma), as a phosphatase inhibitor. Each fraction containing a single 330-kDa component by electrophoresis in SDS-reducing gels was pooled for further use (22–24, 31).To isolate individual forms of TG, fractions of the Sephacryl S-300 column eluting with markers defining 27, 19, or 12 S TG moieties were subjected to high pressure gel permeation chromatography (HPGPC) using a 7.5 × 300-mm TSK-4000 SW column (Tosohaas, Montgomeryville, PA). A Gilson HPLC system (Gilson, Inc., Middletown, WI), pumping prefiltered, degassed phosphate-buffered saline, pH 7.5, at 0.5 ml/min, was used to resolve 0.2-ml samples containing 10 mg of TG protein; detection was by dual wavelength 280/256 monitoring.

Autophosphorylated TG was prepared by incubating phenyl phosphate-free TG at 30 °C for 15 min in 10 mM Tris-Cl, pH 8.0, containing 10 mM $MgCl_2$ and 100 μM ATP (37). The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and the sample dialyzed against Tris-Cl, pH 7.6. TG phosphorylated by treatment with protein kinase A (PKA) was prepared (37) by incubating TG at 30 °C for 15 min in 10 mM Tris-Cl, pH 7.5, containing 10 mM $MgCl_2$ 200 μM ATP, and the catalytic subunit of cAMP-dependent PKA (New England Biolabs, Beverly, MA). The reaction was stopped by adding a heat stable inhibitor of cAMP-dependent protein kinase (New England Biolabs); and the reaction dialyzed as above.

AsialoTG was prepared (23, 24) by incubating 30 mg/ml TG, with 100 units/ml normal or heat-inactivated *Clostridium perfringens* neuraminidase (Sigma) for 24 h in 0.1 M sodium acetate, pH 5.5, and at 37 °C. Heat inactivation was for 10 min at 80 °C. The TG was then isolated by HPGPC as described above and added to cells transfected with promoter chimeras as detailed below.

Acid Hydrolysis and Analysis of Phosphoamino Acids—Phosphorylated TG preparations were dialyzed against 50 mM ammonium acetate, lyophilized, and acid-hydrolyzed *in vacuo* in 6 N HCl for 2 h at 110 °C, as described (31, 37, 40). The hydrolyzed samples were twice diluted with 50 mM ammonium bicarbonate, pH 7.8, lyophilized, then dissolved in 20 μl of electrophoresis buffer, pyridine/acetic acid/water (10/100/890, v/v/v) at pH 3.5, containing 0.1 μM each of phosphoserine and phosphothreonine. Electrophoresis was on 0.25-mm cellulose thin layer plates at 1000 V for 60 min at 10 °C (31, 37). Standards were visualized by ninhydrin; autoradiographs were developed at −70 °C with intensifying screens. Alkali treatment of the ^{32}P-labeled TG was performed in 1 N NaOH at room temperature for 30 min (31). After alkali hydrolysis, the TG was precipitated with 10% trichloroacetic acid for measurement of residual radioactivity.

Antibodies—Anti-rat TG and anti-bovine TG sera were those previously described and characterized (22–24, 31). Rabbit antiserum to the RHL-1 subunit of the rat thyroid ASGPR was prepared using purified, His-tagged, recombinant protein prepared in *Escherichia coli* transfected with RHL-1 cDNA encoding the carbohydrate recognition domain.[2] The anti-RHL-1 specifically recognizes the RHL-1 subunit of the rat thyroid ASGPR.[2]

RNA Isolation and Northern Analyses—Cells were washed with fresh 6H or 5H medium before the noted concentrations of TG or other agents were added. RNA was prepared using a Total RNA Isolation kit (5 Prime→3 Prime, Inc., Boulder City, CO) with minor modifications of the manufacturer's protocol (17–19). RNA samples (20 μg) were run on denatured agarose gels, blotted, UV cross-linked, and hybridized as described (17–19). The probes for TG, TPO, and TTF-1 were labeled with [^{32}P]dCTP (17–19). The rat β-actin probes was prepared by reverse transcription-polymerase chain reaction using FRTL-5 cell poly(A)$^+$ RNA (4, 17, 19). In select experiments, 30 nM okadaic acid (Sigma) was added to cells 30 min before the addition of 1 mg/ml 19 or 27 S TG. Quantitation used a BAS-1500 bioimaging analyzer (Fuji Medical Systems, Stamford, CT).

Transient Expression Analysis—FRTL-5 cells in 5H or 6H medium were exposed to 5 μg of each reporter gene chimera and 2 μg of pSVGH using a DEAE procedure (17–19). pSVGH was used to measure trans-

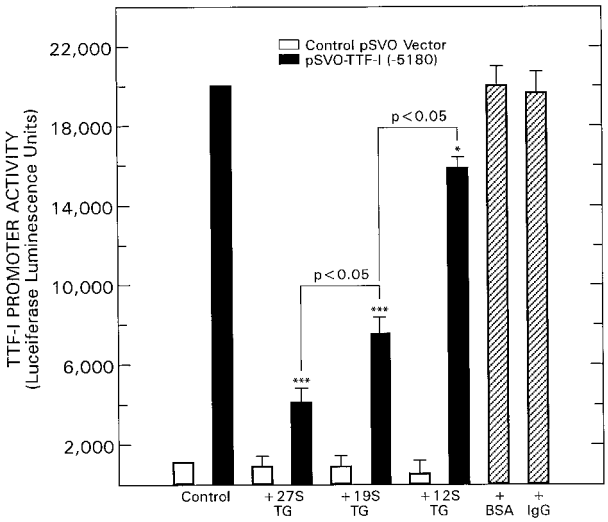

FIG. 2. **Ability of 27, 19, or 12 S TG to decrease TTF-1 promoter activity in FRTL-5 thyroid cells.** FRTL-5 cells in 6H medium were transfected with 5 μg of the pSVO-TTF-1(−5180)-luciferase chimera (*black bars*) or a control pSVO vector (*open bars*). After 24 h, cells were washed and exposed to 1 mg/ml 27, 19, or 12 S TG isolated as described in Fig. 1*B*, 1 mg/ml BSA, or 1 mg/ml bovine immunoglobulin G (*IgG*). After 48 h, promoter activity was measured and normalized for transfection efficiency. Values are expressed in arbitrary luminescence units; data are the mean ± S.D. of three different experiments in duplicate. *One* and *three asterisks* represent a significant TG-induced decrease by comparison to controls with no added ligand at $p < 0.05$ or $p < 0.01$, respectively.

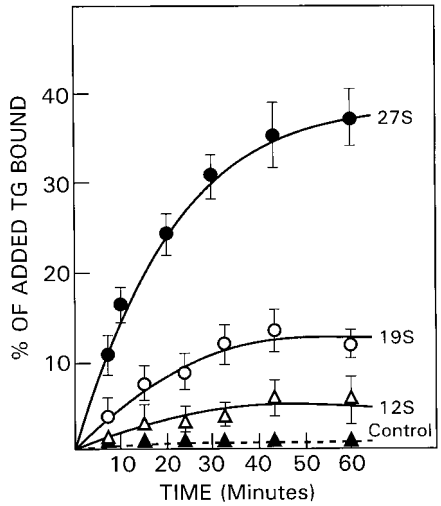

FIG. 3. **Binding of 27, 19, or 12 S radioiodinated TG to FRTL-5 thyroid cells.** Binding incubations included the same amounts of TG protein and cpm; assays were performed as described under "Materials and Methods." Controls with no thyroid cells or with 10^{-6} M unlabeled TG (- - -) exhibited no significant radiolabeled TG binding. Data are the mean ± S.D. of three experiments, each performed in triplicate.

fection efficiency (41). Cells were returned to the 5H or 6H medium for 24 h, at which time they were exposed to fresh medium containing the agents to be tested. Chloramphenicol acetyltransferase (CAT) or luciferase activity was measured after 48 h (17–19). The coefficient of variation of transfection efficiency in 24 different experiments was 8.7%.

The preparation and properties of the class I-CAT chimera have been detailed (17–19). TPO-luciferase constructs were obtained from TPO-CAT constructs by placing the upstream sequence into the luciferase reporter plasmid, pSV0AL-AΔ5, which was also used to measure transfection efficiency (17). Rat TTF-1(T/EBP)-luciferase constructs were prepared by polymerase chain reaction amplification of various lengths of the TTF-1 gene upstream sequences using a rat genomic clone as template (17, 19). In some experiments, FRTL-5 cells transfected with the different chimeras and incubated for 24 h in 6H medium were treated with 100 units/ml normal or heat-inactivated *Clostridium perfringens* neuraminidase (Sigma) for 24 h in 6H medium. Cells were then extensively washed with fresh 6H medium, and 19 S TG (1 mg/ml) was added.

Iodination of Thyroglobulin and Thyroglobulin Binding—The different thyroglobulin preparations were radioiodinated using a lactoperoxidase procedure (22–24). Free iodide was removed by gel filtration on a 9 × 10-cm Sephadex G25 column equilibrated with 0.1% crystalline bovine serum albumin (BSA) and 0.1 M potassium phosphate, pH 7.2 (22–24). All procedures were at room temperature; radioactivity incorporated averaged 25 ± 4 μCi/μg protein (22–24). Assays to measure binding to FRTL-5 cells in 24-well plates included 100,000 cpm of the radioiodinated TG in a 350-μl reaction volume containing 0.025 M Tris acetate, pH 7.0, 2.5% BSA, 0.02 M $CaCl_2$, 220 mM sucrose, and 50,000 cells. Unlabeled bovine TG or IgG (1×10^{-6} M) was added to control wells. Incubations were at 37 °C for the times noted and were stopped by the addition of 2 ml of ice-cold 0.025 M Tris acetate, pH 7.0, containing 2.5% BSA, 0.02 M $CaCl_2$, and 220 mM sucrose. Cells were lysed with 1 N NaOH and radioactivity measured in a γ counter (22–24). Protein concentration was determined by Bradford's method (Bio-Rad); recrystallized BSA was the standard.

Western Blotting—Subconfluent FRTL-5 cells grown in complete 6H medium were washed twice with phosphate-buffered saline, pH 7.4, lysed in Tris-SDS-β-mercaptoethanol buffer (Owl Scientific, Woburn, MA), and sonicated twice for 15 s to reduce viscosity. The whole cell extracts (20 μg/lane) were boiled in lysis buffer for 5 min and subjected to 10% SDS-gel electrophoresis (Novex, San Diego, CA) in replicate. After electroblotting on nitrocellulose membranes, membranes were blocked with TBS-T (10 mM Tris-HCl, pH 8.0, 0.2% Tween 20, 150 mM NaCl) containing 10% nonfat dry milk and separated into individual lanes. Individual lanes were preincubated for 2 h with 19 S bovine TG, autophosphorylated 19 S bovine TG, 27 S bovine TG, or bovine IgG, each at 1 mg/ml, or with a 1:1000 dilution of preimmune or immune serum against the RHL-1 subunit of the rat thyroid ASGPR. All the above steps were at 4 °C. Strips incubated with TG were washed twice with TBS-T and incubated with anti-bovine TG in blocking buffer for 2 h at room temperature. Finally, all strips were washed with TBS-T, then incubated with horseradish peroxidase-conjugated, donkey anti-rabbit IgG (Amersham Pharmacia Biotech) at a 1:2000 dilution for 1 h at room temperature. Membranes were washed with TBS-T and ECL (Amersham Pharmacia Biotech) detection performed.

Materials—Highly purified bovine TSH (30 units/mg) was obtained from the hormone distribution program of the NIDDK, National Institutes of Health, Bethesda, MD. The source of other materials was Sigma, unless otherwise noted.

Statistical Significance—All experiments were repeated at least three times using different batches of cells. Values are the mean ± S.D. of these experiments. Significance between experimental values was determined by two-way analysis of variance and is significant if P values were <0.05.

RESULTS

The Action of TG to Suppress Thyroid-restricted Gene Expression Involves Binding to the ASPGR on the Apical Membrane—In our previous reports (17–19), we used highly purified 19 S follicular TG to evaluate its suppressive effect on TG, TPO, and NIS gene expression in rat FRTL-5 thyroid cells and on suppression of TTF-1, Pax-8, and TTF-2, the thyroid-restricted transcription factors that control TG, TPO, and NIS expression in thyrocytes. Although 19 S TG is the predominant form of TG in the follicular lumen, as evidenced by gel filtration chromatography of salt extracted TG under nondenaturing conditions, a significant amount of 27 and 12 S TG can exist in the extracts (Fig. 1*A*). Each of these was separated by HPGPC (Fig. 1*B*) and shown to contain a single 330-kDa protein species on overloaded SDS-reducing gels (data not shown). The 27 S TG moiety was a significantly more effective suppressor at comparable TG concentrations, 1 mg/ml, than 19 or 12 S TG (Fig. 2). There was no suppression by control proteins, BSA or IgG, in this or previous experiments (Fig. 2; Refs. 17–19).

The ability of each TG moiety to inhibit TTF-1 promoter activity reflected their ability to bind to FRTL-5 cell thyroid cells, *i.e.* 27 S > 19 S > 12 S (Fig. 3). Thus, radioiodinated TG binding to FRTL-5 cell thyroid cells had the same relative

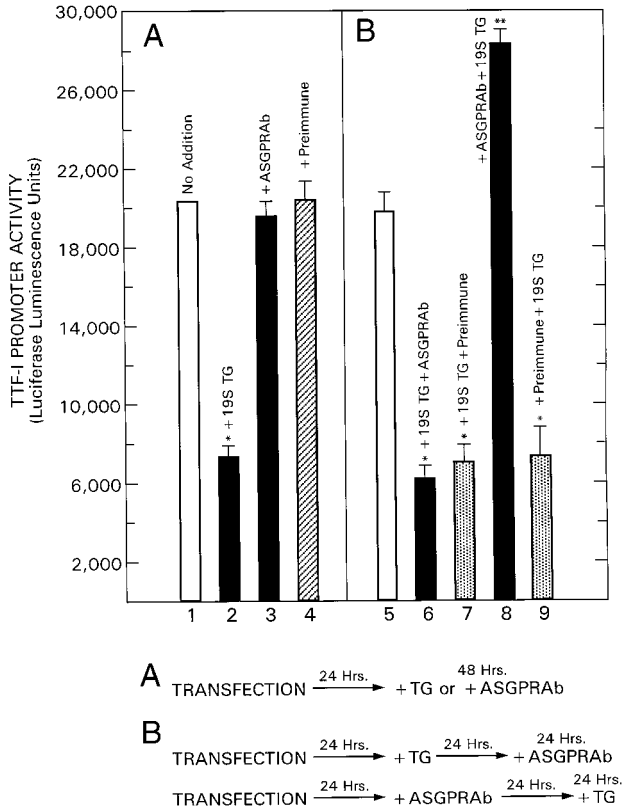

FIG. 4. **Ability of an antibody against the recombinant RHL-1 subunit of the rat thyroid asialoglycoprotein receptor (ASGPR) to prevent 19 S suppression of TTF-1 promoter activity in FRTL-5 thyroid cells.** FRTL-5 cells in 6H medium were transfected with 5 μg of the pSVO-TTF-1(−5180)-luciferase chimera (*open bars*) in *panels A* and *B*. In *A*, 24 h after transfection cells were exposed for 48 h to 1 mg/ml 19 S TG or 1 mg/ml IgG from an antisera directed against the ASGPR or 1 mg/ml IgG from preimmune serum, at which time promoter activity was measured and normalized for transfection efficiency. In *B*, 24 h after transfection, cells were exposed to 0.1 mg/ml ASGPR or preimmune IgG for 24 h then to 1 mg/ml 19 S TG for a subsequent 24 h (*bars 8* and *9*, respectively). Alternatively, 24 h after transfection, cells were exposed to 1 mg/ml 19 S TG for 24 h then to 0.1 mg/ml ASGPR or preimmune IgG for 24 h (*bars 6* and *7*, respectively). Promoter activity was measured after the 48 h incubation with the 19 S TG and the IgG, normalized for transfection efficiency, and values expressed in arbitrary luminescence units. Data are the mean ± S.D. of three different experiments in duplicate. *One asterisk* represents a significant TG-induced decrease by comparison to controls with no added ligand at $p < 0.02$; *two asterisks* represent a significant, $p < 0.01$, loss in 19 S TG suppression activity.

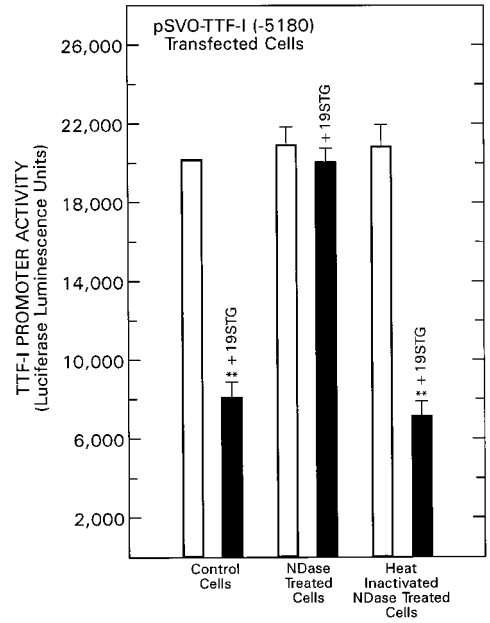

FIG. 5. **Effect of neuraminidase treatment of FRTL-5 cells on the ability of 19 S TG to suppress TTF-1 promoter activity in FRTL-5 thyroid cells.** FRTL-5 cells in 6H medium were transfected with 5 μg of the pSVO-TTF-1(−5180)-luciferase chimera (*open bars*). Twenty-four hours after transfection, cells were exposed to normal or heat inactivated neuraminidase as described (see "Materials and Methods"); and then for 48 h to 19 S TG (*black bars*), at which time promoter activity was measured and normalized for transfection efficiency. Values expressed in arbitrary luminescence units; data are the mean ± S.D. of three different experiments in duplicate. *Two asterisks* represent a significant, $p < 0.02$, decrease in TTF-1 promoter activity by comparison to controls.

order, as a function of the TG moiety tested, 27 S > 19 S > 12 S (Fig. 3), as did 27, 19, or 12 S suppression of TTF-1 promoter activity (Fig. 2). This suggested that suppression might involve a cell surface receptor.

We had previously identified a TG binding site on thyrocyte membranes and shown its binding properties were similar to that of the ASGPR on liver membranes (22–25). This receptor has recently been demonstrated to be on the apical membrane of polarized thyrocytes.[2] To see if the thyroid ASGPR was involved in the suppression process, we evaluated the effect of a specific antibody against the recombinant RHL-1 subunit of the rat thyroid ASGPR on TG suppression activity (Fig. 4). An IgG preparation from antiserum to the rat thyrocyte ASGPR (ASGPRAb) did not itself significantly decrease TTF-1 promoter activity nor did IgG from preimmune sera, by comparison to 19 S TG (Fig. 4*A*, *black bar 3* and *hatched bar 4*, respectively, *versus black bar 2*). However, when the ASGPRAb was incubated with cells for 24 h before 19 S TG was added, the suppressive effect of 19 S TG was lost (Fig. 4, *B*, *black bar* 8 *versus A*, *black bar 2*). IgG from preimmune sera did not, in contrast, affect the ability of 19 S TG to suppress TTF-1 promoter activity when it was comparably preincubated with the cells before 19 S TG was added (Fig. 4, *B*, *hatched bar* 9 *versus B, black bar 8* or *A*, *black bar 2*). Addition of the ASGPRAb after preincubation of the 19 S TG with the cells for 24 h did not reverse the ability of 19 S TG to inhibit promoter activity (Fig. 4*B*, *black bar 6 versus 8*) but, rather, had the same action as IgG from preimmune sera that had been added under the same conditions (Fig. 4*B*, *hatched bar 7*). These experiments could be duplicated using 27 S TG as the suppressor (data not shown). These data indicated that the thyroid ASGPR, which is on the apical membrane of polarized thyrocytes,[2] was involved in the suppression process.

Neuraminidase treatment of membranes can inactivate the TG binding activity of the thyroid or liver ASGPR (22). This reflects the uncovering of galactose residues on surface glycoproteins, including the ASGPR itself, and binding of these cell surface glycoproteins to the lectin-like binding site of the ASGPR, thereby blocking exogenous asialoglycoprotein binding (22, 42). Neuraminidase treatment of the FRTL-5 cells eliminated the ability of 19 S TG (Fig. 5) or 27 S TG (data not shown) to suppress TTF-1 promoter activity (Fig. 5). In contrast, treating cells with heat-inactivated neuraminidase had no effect on TG suppressive activity (Fig. 5); and neuraminidase treatment of 19 or 27 S TG (see "Materials and Methods") had no effect on their ability to suppress TTF-1 promoter activity, but rather enhanced suppression by about 20% in each case (data not shown).

The different relative abilities of 27, 19, and 12 S TG to suppress thyroid-restricted gene expression was also evident when TG, NIS, TSHR, or TPO promoter activities were measured. This is illustrated using a pSVO-TPO(−1362)-luciferase

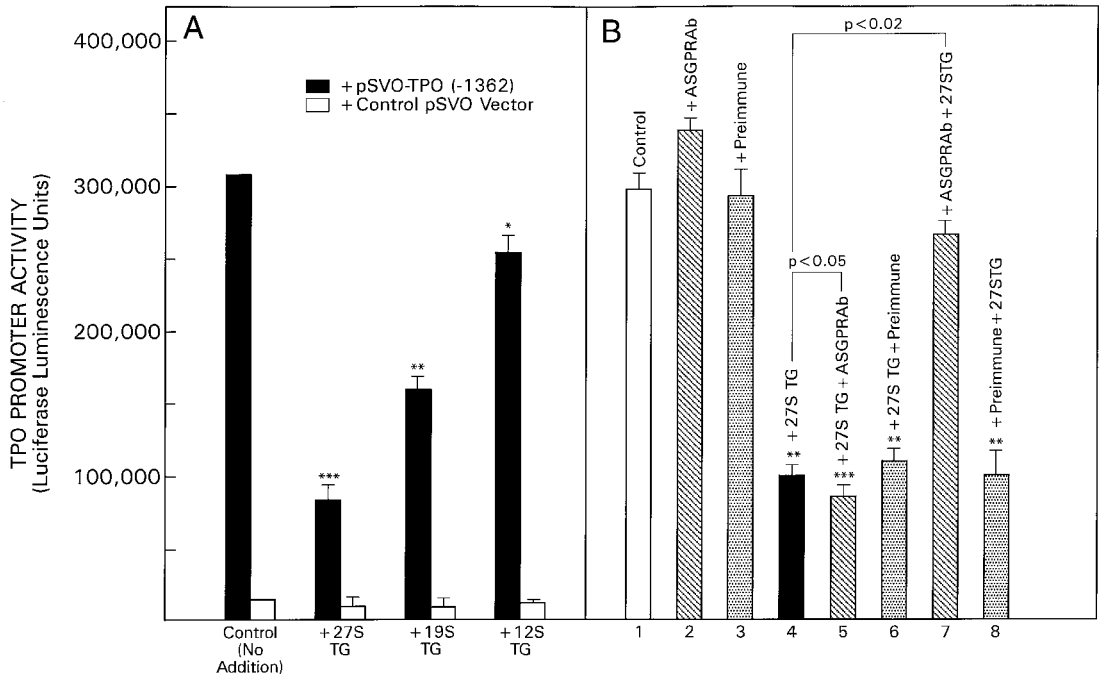

FIG. 6. **Ability of 27 S, 19 S, or 12 S TG to decrease TPO promoter activity in FRTL-5 thyroid cells (*A*) and the ability of an antibody against the recombinant RHL-1 subunit of the rat thyroid ASGPR to prevent 27 S suppression of TPO promoter activity in FRTL-5 thyroid cells (*B*).** In *A*, FRTL-5 cells in 6H medium were transfected with 5 μg of the pSVO-TPO(−1362)-luciferase chimera (*black bars*) or a control pSVO vector (*open bars*). After 24 h, cells were washed and exposed to 1 mg/ml 27, 19, or 12 S TG as in Fig. 2. After 48 h, promoter activity was measured and normalized for transfection efficiency. In *B*, FRTL-5 cells in 6H medium were transfected with 5 μg of the pSVO-TPO(−1362)-luciferase chimera as in *panel A*. Twenty-four hours after transfection, the cells were exposed for 48 h to 1 mg/ml 27 S TG (*bar 4*) or 1 mg/ml IgG from an antiserum directed against the ASGPR or 1 mg/ml IgG from preimmune serum (*bars 2* and *3*, respectively) at which time promoter activity was measured and normalized for transfection efficiency. In experiments performed simultaneously, cell which had been transfected 24 h earlier were exposed to 0.1 mg/ml ASGPR or preimmune IgG for 24 h then to 1 mg/ml 27 S TG for a subsequent 24 h (*bars 7* and *8*, respectively). Alternatively, 24 h after transfection, cells were exposed to 1 mg/ml 27 S TG for 24 h then to 0.1 mg/ml ASGPR or preimmune IgG for 24 h (*bars 5* and *6*, respectively). Promoter activity was measured after the 48-h incubation with the 27 S TG and the IgG, normalized for transfection efficiency, and values expressed in arbitrary luminescence units. Data are the mean ± S.D. of three different experiments in duplicate. *One asterisk* represents a significant TG-induced decrease by comparison to controls with no added ligand at $p < 0.05$; *two asterisks* represent a significant, $p < 0.02$, decrease; *three asterisks* represent a significant $p < 0.01$ decrease.

chimera (Fig. 6*A*). Similar data were also obtained with the antibody to the ASGPR, as again illustrated with the TPO promoter-luciferase chimera (Fig. 6*B*). Thus, an IgG preparation from the ASGPR antisera, ASGPRAb, did not itself decrease TPO promoter activity nor did IgG from preimmune sera (Fig. 6*B*, *bars 2* and *3 versus bar 1*). When, however, the ASGPRAb was incubated with cells for 24 h before TG was added, the suppressive effect was significantly attenuated (Fig. 6*B*, *bar 7 versus bar 4*). This was not the case with IgG from preimmune sera (Fig. 6*B*, *bar 8*). Addition of the ASGPRAb or preimmune IgG after preincubation of the TG with the cells for 24 h again did not reverse the TG inhibition of promoter activity (Fig. 6*B*, *bars 5* and *6*, respectively). We used 27 S TG as the suppressor in these experiments, but data with 19 S TG were similar.

In our previous report (17), we showed that TG suppression was specific for thyroid-restricted genes. One part of that evidence was the observation that 19 S TG increased, rather than decreased, major histocompatibility (MHC) class I gene expression. The ASGPRAb prevented the increase in MHC class I activity when preincubated with FRTL-5 cells 24 h before 19 S TG was added (Fig. 7, *black bar 4 versus black bar 3*). This was not the case for preimmune IgG (Fig. 7, *hatched bar 7 versus black bar 4*) and was again not the case if the TG was added before the ASGPRAb (Fig. 7, *black bar 5 versus black bar 4*). Studies using 27 S TG were the same.

In summary, the ability of TG to suppress thyroid restricted-gene expression and increase MHC class I expression, which we previously described (17–19), involves TG binding to the ASGPR.

The Action of TG to Suppress Thyroid-restricted Gene Expression Is Reversed by Okadaic Acid Treatment of Cells or by Phosphorylation of Serine/Threonine Residues on TG—The ASGPR on rat liver cells is known to contain phosphotyrosine, phosphoserine, and phosphothreonine residues (34–36, 43, 44). Phosphotyrosine residues have been implicated in ATP-dependent receptor inactivation of endocytosis, whereas alterations in phosphoserine and phosphothreonine residues have not been reported to affect endocytosis (34–36, 43, 44). Previous work has also identified phosphate residues on carbohydrate units (mannose 6-phosphate), tyrosine, and serine/threonine residues of the TG molecule (25, 28–32). Additionally, evidence has been presented that TG has an associated kinase activity and an ability to autophosphorylate serine residues in the presence of ATP (37).

When rat FRTL-5 thyroid cells were treated with okadaic acid, a potent inhibitor of serine/threonine-specific protein phosphatases 1 and 2A (45–48), the ability of 19 or 27 S TG to suppress TTF-1 or TPO promoter activity was abolished, despite the absence of an affect on control TTF-1 (Fig. 8*A*) or TPO (Fig. 8*B*) promoter activity. There was no effect of okadaic acid on transfections with the pSVO vector alone (data not shown). The effect of okadaic acid was evident when intrinsic TTF-1 (Fig. 9), TG (Fig. 10*A*), or TPO (Fig. 10*B*) RNA levels were measured, *i.e.* okadaic acid treatment eliminated the ability of 27 or 19 S TG to suppress thyroid-restricted gene expression.

One possibility we considered was that the okadaic acid was modulating serine/threonine residues on the ASGPR, increasing their phosphorylation state, and thereby eliminating the

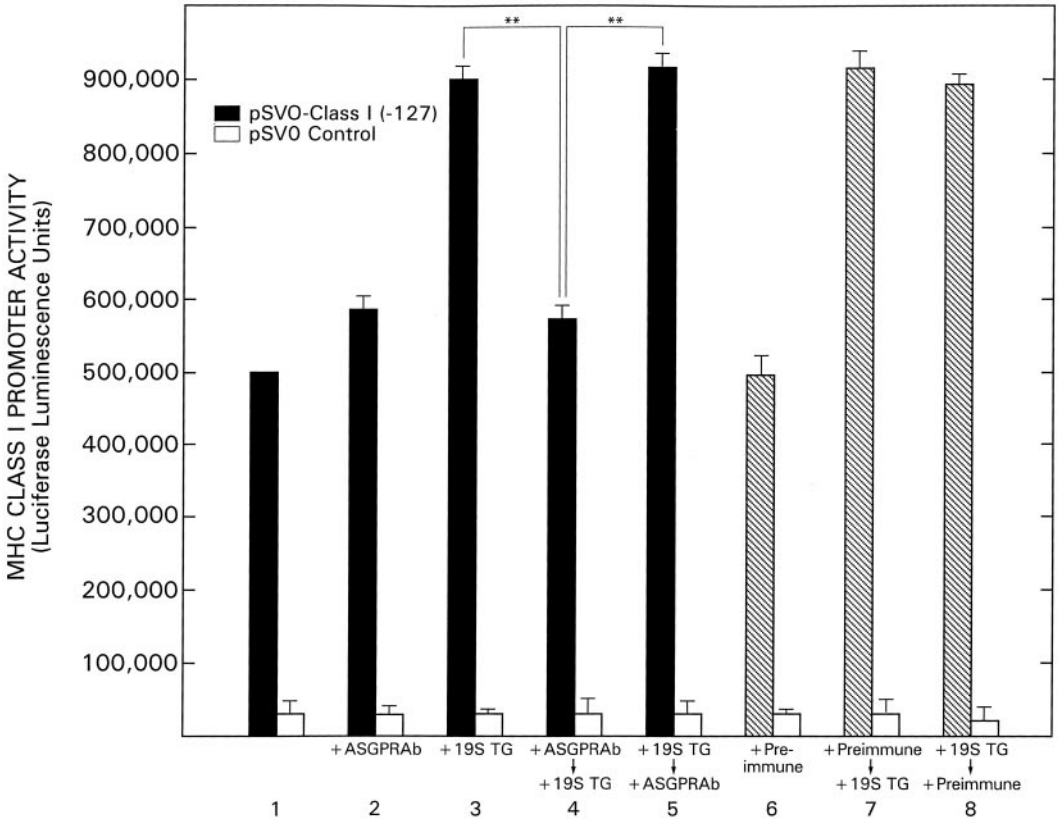

FIG. 7. **Ability of 19 S TG to increase MHC class I promoter activity in FRTL-5 thyroid cells and the ability of an antibody against the recombinant RHL-1 subunit of the rat thyroid ASGPR to prevent the 19 S TG-induced increase in that activity.** FRTL-5 cells in 6H medium were transfected with 5 μg of the pSVO-class I(−127)-luciferase chimera (*black bars*) or a control pSVO vector (*open bars*). After 24 h, cells were washed and exposed to 1 mg/ml 19 S TG as in Fig. 6 for 48 h. After promoter activity was measured and normalized for transfection efficiency, the activity compared with controls with no TG (*lane 3 versus lane 1*). Alternatively, cells were transfected with 5 μg of the pSVO-class I(−127)-luciferase chimera and 24 h after transfection, the cells were exposed for 48 h to 1 mg/ml IgG preparation from an antisera directed against the ASGPR (*lane 2*) or 1 mg/ml IgG from preimmune serum (*bar 6*). In experiments performed simultaneously, cells that had been transfected 24 h earlier were exposed to 0.1 mg/ml ASGPR or preimmune IgG for 24 h then to 1 mg/ml 19 S TG for a subsequent 24 h (*bars 4* and *7*, respectively) or were exposed to 1 mg/ml 19 S TG for 24 h then to 0.1 mg/ml ASGPR or preimmune IgG for 24 h (*bars 5* and *8*, respectively). Promoter activity was measured after the 48-h incubations with the 19 S TG and the IgG, normalized for transfection efficiency, and values expressed in arbitrary luminescence units. Data are the mean ± S.D. of three different experiments in duplicate. *Two asterisks* represent a significant, $p < 0.02$, loss in 19 S TG increased class I activity by ASGPR antibody preincubation.

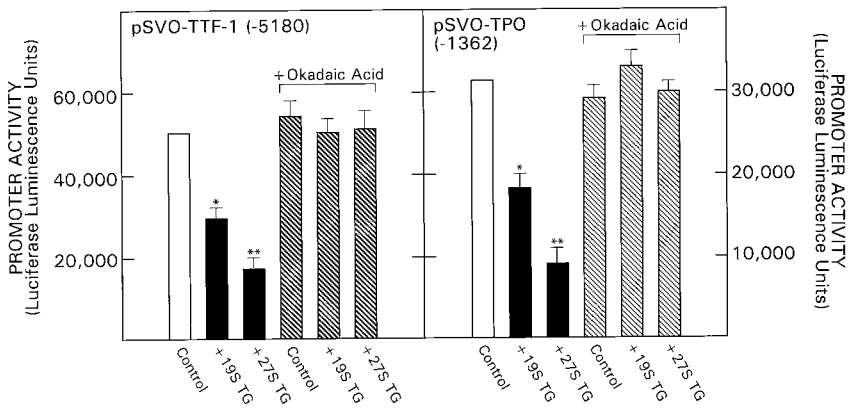

FIG. 8. **Effect of okadaic acid treatment of FRTL-5 cells on the ability of 19 or 27 S TG to suppress TTF-1 (*A*) or TPO (*B*) promoter activity in FRTL-5 thyroid cells.** FRTL-5 cells in 6H medium were transfected with 5 μg of the pSVO-TTF-1(−5180) or pSVO-TPO(−362)-luciferase chimeras (*open bars*). Twenty-four hours after transfection, cells were exposed to 1 mg/ml 19 or 27 S TG in the absence (*black bars*) or presence (*hatched bars*) of 30 nM okadaic acid as described under "Materials and Methods." Promoter activity was measured after 48 h and normalized for transfection efficiency. Values are expressed in arbitrary luminescence units; data are the mean ± S.D. of three different experiments in triplicate. *One asterisk* represents a significant, $p < 0.05$, TG-induced decrease by comparison to controls; *two asterisks* represent a significant, $p < 0.02$, decrease.

suppressive action of TG. Alternatively, we considered the possibility that the okadaic acid was modulating serine/threonine residues on TG itself. To test the latter possibility, we phosphorylated 19 S TG with PKA or autophosphorylated purified 19 S TG in the presence of ATP. The TG phosphorylated by either method no longer suppressed TTF-1 (Fig. 9), TG (Fig. 10*A*), or TPO (Fig. 10*B*) RNA levels. Of particular interest, however, was the observation that phosphorylated TG actually

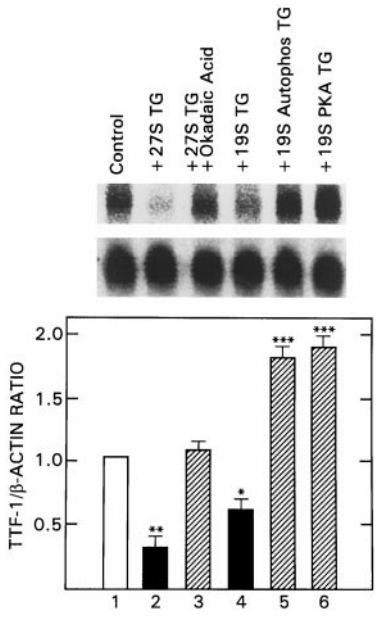

FIG. 9. **Effect of okadaic acid treatment of FRTL-5 cells or of phosphorylation of follicular TG on the ability of follicular TG to decrease endogenous TTF-1 RNA levels.** FRTL-5 cells in 6H medium were exposed to 1 mg/ml 27 S TG in the absence (*lane 2*) or presence (*lane 3*) of 30 nM okadaic for 48 h. Alternatively they were exposed to native 19 S TG or 19 S TG that had been autophosphorylated or phosphorylated with PKA for 48 h. RNA was extracted and sequential Northern analysis performed as described under "Materials and Methods" using a TTF-1 or β-actin probe. The *top* of the figure depicts a representative Northern analysis; the *bottom* depicts the ratio of the TTF-1/β-actin RNAs measured in four independent experiments and expressed as the mean ± S.D. Quantitation was with a BAS-1500 bioimaging analyzer (Fuji Medical Systems). *One asterisk* represents a statistically significant decrease of $p < 0.05$ by comparison to control TTF-1 RNA levels, and *two asterisks* a statistically significant decrease of $p < 0.02$. *Three asterisks* represent a statistically significant $p < 0.01$ increase by comparison to both controls and the ability of normal follicular TG to suppress TTF-1 RNA levels.

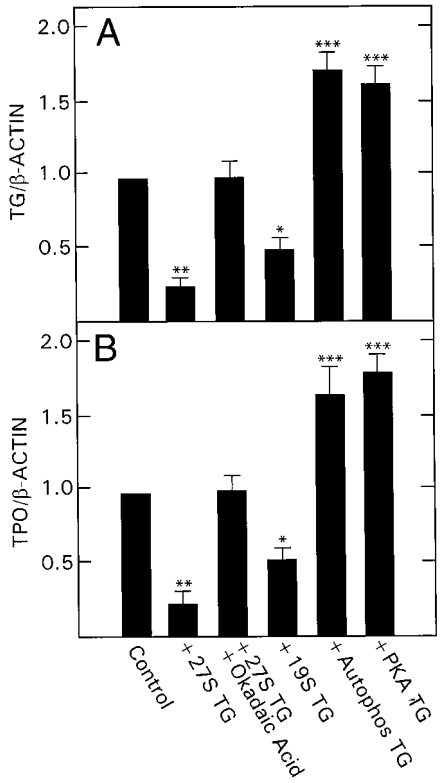

FIG. 10. **Effect of okadaic acid treatment of FRTL-5 cells or of phosphorylation of follicular TG on the ability of follicular TG to decrease endogenous TG (*A*) or TPO (*B*) RNA levels.** FRTL-5 cells in 6H medium were exposed to 1 mg/ml 27 S TG in the absence (*lane 2*) or presence (*lane 3*) of 30 nM okadaic for 48 h. Alternatively they were exposed to native 19 S TG or 19 S TG that had been autophosphorylated or phosphorylated with PKA for 48 h. RNA was extracted and sequential Northern analysis performed as described under "Materials and Methods" using a TG, TPO, and β-actin probes. The ratio of the TG or TPO to β-actin RNA was quantitated with a BAS-1500 bioimaging analyzer (Fuji Medical Systems). Data from four independent experiments were averaged expressed as the mean ± S.D. *One asterisk* represents a statistically significant decrease of $p < 0.05$ by comparison to control TG or TPO RNA levels, and *two asterisks* a statistically significant decrease of $p < 0.02$. *Three asterisks* represent a statistically significant $p < 0.01$ increase by comparison to both controls and the ability of normal follicular TG to suppress TG or TPO RNA levels.

increased TTF-1 RNA levels nearly 2-fold over controls, in contrast to okadaic acid treatment of the cells, which merely abolished the TG suppressive effect (Figs. 8–10). Optimal increases of all thyroid-restricted RNAs were with 0.1 mg of autophosphorylated TG (data not shown) by comparison to the 1–10 mg of TG needed for optimal suppression (17).

Autophosphorylation of TG has been reported to phosphorylate phosphoserine residues only (37). We confirmed that phosphoserine residues were formed during the autophosphorylation reaction. Thus, when we used [^{32}P]ATP and subjected the autophosphorylated 19 S TG to acid hydrolysis and phosphoamino acid analysis as described (see "Materials and Methods"; Refs. 31, 37, and 40), only phosphoserine and not phosphotyrosine or phosphothreonine residues were detected (data not shown).

The absence of an effect of okadaic acid or phosphorylated TG on β-actin RNA levels was duplicated with glyceraldehyde-3-phosphate dehydrogenase RNA levels, *i.e.* the effect appeared to be specific as is the case for TG suppression of thyroid-restricted gene expression (17). There was no effect on 19 or 27 S TG suppression of TTF-1, TPO, or TG RNA levels when the autophosphorylation reaction components without TG were incubated together for the same time, EDTA added, the mixture dialyzed against Tris-Cl, pH 7.6, and then added with 19 or 27 S TG. Similarly, addition of the reaction components used in the PKA phosphorylation reactions, which were treated with the heat-stable inhibitor of cAMP-dependent PKA and then dialyzed, had no effect on 19 or 27 S TG suppression of TTF-1, TPO, or TG RNA levels.

The phosphorylated TG still binds to the thyroid ASGPR. Thus, when FRTL-5 whole cell extracts were subjected to SDS-gel electrophoresis and blotted on nitrocellulose membranes (Fig. 11), ECL staining identified a 42-kDa protein able to bind 19 S autophosphorylated TG or 19 S TG (Fig. 11, *lanes 3* and *4*) but not IgG (*lane 5*). The 42-kDa protein is the size of the RHL-1 subunit of the ASGPR[2] and has the same mobility as a protein incubated with the anti-RHL-1 subunit of the thyroid ASGPR (Fig. 11, *lane 1*). Control preimmune sera did not result in identification of the 42-kDa protein (Fig. 11, *lane 2*).

DISCUSSION

The thyroid follicle is the functional unit of the thyroid gland (1–3, 20, 21, 25, 49). Thyroid cells secrete and store TG into its lumen; they also concentrate and secrete iodide into the follicular lumen, where TPO and H_2O_2 iodinate the TG tyrosine residues and contribute to iodotyrosine coupling to form thyroid hormones. Thyroid hormones are secreted into the blood stream, after TG is transported to lysosomes and degraded. This complex process is controlled by TSH and insulin/IGF-1 (1–3, 20, 21, 25, 50). TSH and insulin/IGF-1 regulate the expression and activity of the thyroid-restricted transcription factors, TTF-1, TTF-2, and Pax-8, which are critical for the

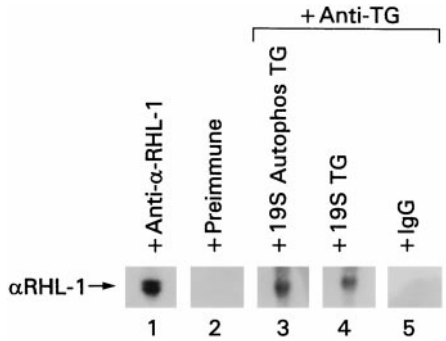

FIG. 11. **Western blot of protein which binds TG and autophosphorylated TG in FRTL-5 thyrocytes.** Subconfluent FRTL-5 cells grown in complete 6H medium were washed with phosphate-buffered saline, lysed in Tris-SDS-β mercaptoethanol buffer, and sonicated twice to reduce viscosity. The whole cell extracts were boiled for 5 min, and 20 μg/lane was subject to 10% SDS-gel electrophoresis in replicate. After electroblotting, the nitrocellulose membranes were blocked with TBS-T, separated into individual lanes, and these were preincubated for 2 h with autophosphorylated 19 S bovine TG (*lane 3*), 19 S bovine TG (*lane 4*), or bovine IgG (*lane 5*), each at 1 mg/ml, or with a 1:1000 dilution of immune or preimmune serum against the RHL-1 subunit of the rat thyroid ASGPR (*lanes 1* and *2*, respectively). Strips incubated with TG or IgG were washed twice with TBS-T and incubated with anti-bovine TG in blocking buffer for 2 h at room temperature. Finally, all strips were washed with TBS-T, then incubated with horseradish peroxidase-conjugated, anti-rabbit IgG at a 1:2000 dilution for 1 h. Membranes were washed with TBS-T and ECL staining performed.

thyroid-restricted expression of the TG, TPO, and NIS genes (10–16).

Each thyrocyte is faced with the same levels of TSH and insulin/IGF-1 in the blood stream, yet the functional state of each follicle varies (18, 19, 51–54). Some have high levels of TG and thyroid hormones in their follicular lumen, exhibit high levels of TPO activity, and are surrounded by metabolically active columnar epithelial cells; others are nearly devoid of TG, TPO, and thyroid hormones and are surrounded by flattened and quiescent thyroid cells (18, 19, 51–54). Recent studies suggest that TG accumulated in the follicular lumen partially explains the heterogeneity of follicular function (17, 18). Thus, purified follicular TG can suppress TSH and insulin/IGF-1 increased TTF-1, TTF-2, and Pax-8 expression and suppress, in turn, TSH or insulin/IGF-1-increased TG, TPO, and NIS gene expression as well as activity (17–19). The effect was very specific. It was not duplicated by iodide or thyroid hormones (17, 18), did not involve ubiquitous transcription factors which also regulated the TSHR, TG, or TPO genes (17), and actually increased expression of MHC class I, which is ubiquitously expressed on most cells in the organism (17).

In this new paradigm of thyroid control, TSH, insulin, and/or IGF-1 enhanced thyroid-restricted gene expression and function; follicular TG, a critical product of thyroid-restricted gene expression, acted as a feedback regulator of the hormonal induced increase (17–19). TSH reinitiated the hormone-induced synthetic phase of follicle function by inducing fluid pinocytosis and TG degradation, a rapid phenomenon relative to the TSH and insulin/IGF-1-initiated synthetic phase of follicular function (55). The fundamental question that arose from this new paradigm is how the TG molecule stored in the follicular lumen, could regulate the transcriptional machinery of the cell.

In the present report, we show that binding of TG to the ASGPR on the apical membrane of the thyrocyte, the membrane facing the follicular lumen, is the initial event in the TG suppression process in thyrocytes. We show that 27 S TG binds to thyroid cells better than 19 S or 12 S TG and that this parallels the suppressive action of the follicular TG moieties, 27 S > 19 S > 12 S. We show that a specific antibody to the RHL-1 subunit of the thyroid ASGPR[2] blocks TG-induced suppression. We show that neuraminidase treatment of cells blocks suppression just as it blocks ASGPR-mediated endocytosis and binding (22, 42). The mechanism of the neuraminidase inhibition of whole cell ASGPR activity is indirect; galactose residues exposed on cell glycoproteins block the lectin binding site on the ASGPR and inhibit exogenous TG binding (22, 42).

The role of the ASGPR in receptor-mediated endocytosis of asialoglycoproteins is well known (56–59). The present report establishes an additional role wherein binding of the ligand to the ASGPR regulates transcriptional events in the cell. Whether this function exists in other cells with an ASGPR and whether this involves regulation of tissue-specific or -restricted genes and cell function, *i.e.* in hepatocytes where its endocytotic role is clear, is unknown. Nevertheless, we provide additional insight into the mechanism of TG suppression by the ASGPR; we show that phosphorylation of the ligand, TG, and possibly the ASGPR, are important in suppression.

With respect to the ASGPR, it is known to be a phosphoprotein with phosphorylated serine, threonine, and tyrosine residues (27, 34–36, 43, 44). The phosphotyrosine residues on the RHL-1 subunit of the ASGPR are critical for ATP-mediated inactivation of receptor-mediated endocytosis (44). Altered phosphorylation of phosphoserine and phosphothreonine residues on the ASGPR shifts the distribution of the ASGPRs in the recycling process, but does not alter endocytotic activity (34–36). In this report, we show that phosphoserine and phosphothreonine residues may be important in the transcriptional signaling process, since okadaic acid treatment of cells inhibits the ability of TG to suppress thyroid-restricted gene expression mediated by the ASGPR. Because the ASGPR has phosphoserine and phosphothreonine residues (27, 34–36), it is reasonable to speculate that increases in ASGPR phosphoserine or phosphothreonine residues are associated with loss of TG suppression activity. Less speculative, however, is the importance of phosphoserine residues on TG to suppressive signaling process.

Autophosphorylation of a single serine residue (37) can, apparently, not only eliminate suppression, but also change the signal invoked by TG binding to the ASGPR from suppression to 2-fold enhancement for most of the thyroid-restricted genes. Phosphorylation of this single serine residue on the TG molecule does this without altering TG binding to the ASGPR. The fact that PKA-dependent phosphorylation, which could involve both phosphoserine and phosphothreonine residues, has the same effect as autophosphorylation does not exclude the possibility that PKA-mediated phosphorylation of more than one serine or of phosphothreonine residues will further regulate the suppression process. It is unclear whether the okadaic acid effect on the cells involves a change in TG phosphorylation. Nevertheless, it is clear that TG regulation of gene expression is dynamic and can either counteract or enhance the TSH effect, dependent on the state of serine/threonine phosphorylation of the TG molecule. PhosphoTG formation may not be TSH-induced but, rather, cAMP-regulated (37).

In a preliminary report,[3] we have shown that autophosphorylated TG also increases pendrin gene expression. Pendrin has iodide/chloride porting activity, and may be the apical iodide porter. The ability of autophosphorylated TG to enhance both genes suggests this may be a specific effect of TG to increase iodide uptake and iodide influx into the follicular lumen independent of TSH. We have preliminarily associated

[3] K. Suzuki, A. Mori, L. Ulianich, L. A. Everett, I. Royaux, E. D. Green, and L. D. Kohn, manuscript in preparation.

this activity physiologically with TG from Graves' thyroids,[3] where there is little TG accumulation in the follicular lumen.

Numerous questions are opened by this report. What is the nature of the autophosphorylated TG reaction? Which serine/threonine kinase and serine/threonine phosphatase is involved in the phosphorylation reaction; how are they regulated; and what relationship, if any, exists between TG and ASGPR phosphorylation? How does the ASGPR/TG interaction regulate transcription? What is the signal? Are these phenomena thyroid-restricted or broadly applicable to the ASGPR-ligand interaction on other cells?

Despite these questions, the data herein provide novel insights into the mechanism by which follicular TG regulates thyroid-restricted gene expression. They support the existence of a new paradigm of dynamic thyroid regulation by hormones acting on the basal thyroid membrane via the blood stream and TG acting on the apical membrane from the follicular lumen. They support the idea that TG is not an inert site for thyroid hormone formation, but also a dynamic regulator of thyroid function. The ASGPR is not only involved in receptor-mediated endocytosis but, in addition, its interaction with ligand may induce a signal that controls transcription of tissue-restricted genes.

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Follicular Thyroglobulin (TG) Suppression of Thyroid-restricted Genes Involves the Apical Membrane Asialoglycoprotein Receptor and TG Phosphorylation

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J. Biol. Chem. 1999, 274:25099-25107.
doi: 10.1074/jbc.274.35.25099