Thyroglobulin regulates the expression and localization of the novel iodide transporter solute carrier family 26 member 7 (SLC26A7) in thyrocytes

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Abstract. Solute carrier family 26 member 7 (SLC26A7), identified as a causative gene for congenital hypothyroidism, was found to be a novel iodide transporter expressed on the apical side of the follicular epithelium of the thyroid. We recently showed that TSH suppressed the expression of SLC26A7 and induces its localization to the plasma membrane, where it functions. We also showed that the ability of TSH to induce thyroid hormone synthesis is completely reversed by an autocrine negative-feedback action of thyroglobulin (Tg) stored in the follicular lumen. In the present study, we investigated the potential effect of follicular Tg on SLC26A7 expression and found that follicular Tg significantly suppressed the promoter activity, mRNA level, and protein level of SLC26A7 in rat thyroid FRTL-5 cells. In addition, follicular Tg inhibited the ability of TSH to induce the membrane localization of SLC26A7. In rat thyroid sections, the expression of SLC26A7 was weaker in follicles with a higher concentration of Tg, as evidenced by immunofluorescence staining. These results indicate that Tg stored in the follicular lumen is a feedback suppressor of the expression and membrane localization of SLC26A7, thereby downregulating the transport of iodide into the follicular lumen.

Key words: Thyroid, Thyroglobulin, Solute carrier family 26 member 7 (SLC26A7), Solute carrier family 26 member 4 (SLC26A4), TSH

DIETARY IODIDE is an essential component for the biosynthesis of thyroid hormones (THs). Circulating iodide is concentrated in thyrocytes via solute carrier family 5 member 5 (SLC5A5; sodium/iodide symporter: NIS), which was identified in 1996; SLC5A5 is expressed on the basolateral membrane of thyrocytes [1]. Solute carrier family 26 member 4 (SLC26A4 or pendrin), identified in 1997 as a causative gene for Pendred syndrome, which is characterized by congenital deafness and thyroid goiter [2, 3], is expressed on the apical membrane, on the opposite side of SLC5A5. SLC26A4 is responsible for iodide efflux into the follicular lumen. Since iodide organification is only partially impaired in Pendred syndrome, the presence of additional iodide transporter(s) on the apical side was speculated [4]. Solute carrier family 26 member 7 (SLC26A7), discovered in 2018 as a novel gene responsible for congenital hypothyroidism [5], is located on the apical membrane, the same side as SLC26A4 [6-8]. Although SLC26A7 was initially identified as a Cl⁻/HCO₃⁻ exchanger in the stomach and kidney [9, 10], mRNA expression profiling of human tissues revealed that SLC26A7 is the most abundantly expressed iodide transporter in the thyroid (analyzed by The Human Protein Atlas database: https://www.proteinatlas.org/ENSG00000147606-SLC26A7/tissue). A bicameral culture chamber model and transport/uptake assays using radiiodine suggested that SLC26A7 discharges iodine in the culture medium [8], which is similar to the function of pendrin [11]. Therefore, SLC26A7 is considered a novel iodide transporter responsible for iodide efflux into the follicular lumen.

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Recently, we evaluated the role of TSH on SLC26A7 expression using rat thyroid FRTL-5 cells and showed that TSH significantly suppressed SLC26A7 mRNA and protein expression. Although suppression of SLC26A7 is thought to result in decreased iodide transport activity, we additionally demonstrated that TSH induces localization of SLC26A7 to the plasma membrane, where it functions as a transporter [12].

Thyroglobulin (Tg), a dimeric glycoprotein with a molecular weight of 660,000, is the major protein of the thyroid, and it accumulates in the follicular colloid. The concentration of follicular Tg varies from less than 1 mg/mL to over 600 mg/mL among individual follicles, which is a histological characteristic of thyroid follicles referred to as follicular heterogeneity [13-16]. The primary role of Tg is to serve as a macromolecular substrate for iodine, which binds covalently to Tg tyrosyl residues during TH synthesis [17]. In addition to this role, we showed that follicular Tg regulates the function of individual follicles via transcriptional suppression of the genes indispensable for iodide transport and TH synthesis [6, 15, 16, 18-24]. Such a negative-feedback effect of Tg is strong enough to overcome the ability of TSH to accelerate TH synthesis. In rat thyroid FRTL-5 cells and primary cultures of human thyroid follicular cells, expression of the Tg, Scl26a4, Scl5a5, thyroid peroxidase (Tpo), dual oxidase 2 (Duox2), dual oxidase maturation factor 2 (Duoxa2), thyroid transcription factor 1 (TTF-1 or Nkx2-1), thyroid transcription factor 2 (TTF-2 or Foxe1), and paired box 8 (Pax8) genes was suppressed by follicular Tg in a dose-dependent manner even under maximal TSH stimulation [15, 16, 18-20]. In this study, we investigated the potential effect of Tg on the expression and subcellular localization of SLC26A7 using FRTL-5 cells.

Materials and Methods

Cell culture and treatment
Rat thyroid FRTL-5 cells were cultured in Coon’s modified Ham’s F-12 medium supplemented with 5% bovine serum (Invitrogen, Waltham, MA, USA) and a mixture of six hormones, bovine TSH (1 mU/mL), insulin (10 μg/mL), hydrocortisone (0.36 ng/mL), transferrin (5 μg/mL), somatostatin (10 ng/mL), and glycyll-L-histidyl-L-lysine acetate (2 ng/mL), as described previously [18, 20, 25]. FRTL-5 cells cultured in 6-well plates (Greiner Bio-One, Kremsmünster, Austria) were stimulated with bovine Tg at concentrations of 0, 0.1, 1, 5, or 10 mg/mL. All reagents listed above and bovine Tg were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

Total RNA purification and reverse transcription real-time PCR
Total RNA was purified using the RNaseasy Plus Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) as described previously [12, 22]; briefly, 1 μg total RNA was incubated with a mixture of 2 μL 10× RT buffer, 2 μL 10× RT Random Primers, 1 μL MultiScribe™ Reverse Transcriptase (50 U/μL), and 0.8 μL 25× dNTPs for 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C. Real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) and run on the Thermal Cycler Dice Real Time System III (Takara Bio, Tokyo, Japan) for 30 sec at 95°C, followed by 40 cycles of 5 sec at 95°C and 60 sec at 60°C, and then 1 cycle of 15 sec at 95°C, 30 sec at 60°C, and 15 sec at 95°C for dissociation. Real-time PCR was performed in at least in triplicate, and the relative mRNA levels were evaluated using the ΔΔCt method as described previously [26]. The primer sets used for real-time PCR were as follows: SLC26A7 forward, 5'-TGCTCCCCAATGAACATCC-3'; SLC26A7 reverse, 5'-CAAGCCACCTGTGTCTTGC-3'; Gapdh forward, 5'-ACAGCAACAGGTTGTTGGGAC-3'; Gapdh reverse, 5'-TTTGGGTTGCACGGAACCTT-3'.

Protein extraction and Western blot analysis
Extraction of cellular proteins and Western blot analysis were performed as described previously [16, 20, 21]. Briefly, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₂VO₃, and 1 μg/mL leupeptin. Cells were then scraped using a disposable cell lifer (Corning, Corning, NY, USA), and the lysate was centrifugated at 15,000 g at 4°C for 10 min to recover the supernatant. Protein concentrations were measured by the Lowry method using the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) and Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. Each protein sample (10 μg) was mixed with 4× SDS sample buffer (Invitrogen) and 2-mercaptoethanol (final concentration 0.25%), separated by SDS-PAGE on NuPage 4–12% Bis-Tris gels (Invitrogen), and transferred to nitrocellulose membranes using iBlot 2 Transfer Stacks (Invitrogen). The membrane was washed with 0.1% Tween 20 in PBS (PBST), placed in blocking buffer (PBST containing 5% nonfat milk) for 1 h at room temperature, and then
incubated with mouse anti-SLC26A7 (1:1,000; Novus Biologicals, Centennial, CO, USA) for 2 h at room temperature or mouse anti-β-actin (1:5,000; Cell Signaling Technology) for 30 min at room temperature. After washing with PBST, the membrane was incubated with horseradish peroxidase (HRP)-labelled horse anti-mouse IgG (1:1,000; Cell Signaling Technology) for 1 h at room temperature. The HRP signal was detected by chemiluminescence using ImmunoStar LD reagents (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and the chemiluminescence was visualized using the C-DiGit blot scanner (LI-COR, Lincoln, NE, USA).

**Luciferase reporter gene assay**

pGL3-Basic reporter plasmids harboring the 5’-flanking regions of the human SLC26A7 gene were prepared previously [12]. FRTL-5 cells maintained in basal medium in 6-well plates (Greiner Bio-One) were transfected with 1 μg of the luciferase reporter plasmids using FuGENE HD transfection reagent (Promega, Madison, WI, USA). Tg was added to the basal medium (5 mg/mL final concentration) at 6 h after transfection, and the cells were incubated for 48 h. Cells were washed with ice-cold PBS and lysed in Glo lysis buffer (Promega). The Bright-Glo Luciferase Assay System (Promega) was used to perform the luciferase assay. Luciferase activity was measured using the SpectraMax i3x multi-mode microplate reader (Molecular Devices, San Jose, CA, USA) and standardized based on the corresponding protein concentrations determined using the DC Protein Assay Kit (Bio-Rad Laboratories). pGL3-Control plasmid containing strong SV40 promoter and enhancer sequences was used as an internal standard for promoter activity.

**Immunofluorescence staining of FRTL-5 cells**

After attaching to the bottom of 4-well chamber slides (Thermo Fisher Scientific), FRTL-5 cells were maintained in the absence of TSH for 5 days. Cells were then treated with 1 mU/mL TSH and/or 5 mg/mL Tg. Immunofluorescence staining was performed as described previously [12]. Briefly, cells were fixed using 10% neutral buffered formalin for 10 min at room temperature and then permeabilized with PBS containing 0.1% Triton X-100. After blocking in PBS containing 3% BSA, the slides were incubated with mouse anti-SLC26A7 (1:500; Novus Biologicals) overnight at 4°C, washed with PBST, and then incubated with Alexa Fluor 594-conjugated goat anti-mouse IgG antibody (1:1,000, Thermo Fisher Scientific) and Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (1:1,000, Thermo Fisher Scientific) for 1 h, and then the nuclei were counterstained with Hoechst 33342 (1:1,000) for 10 min at room temperature. The sections were washed in PBST, and cover slips were mounted using Fluorescence Mounting Medium (Dako). Fluorescence was observed under the FV10i-DOC confocal laser scanning microscope (Olympus, Tokyo, Japan).

**Statistical analysis**

Statistical analyses were performed using SPSS (IBM Corp., Armonk, NY, USA). Results are expressed as the mean ± standard deviation (SD). Significant differences were determined by one-way analysis of variance (ANOVA), followed by Dunnett’s post hoc test. P values <0.05 were considered significant.

**Results**

**Follicular Tg suppressed SLC26A7 expression in FRTL-5 cells**

We first examined the potential effect of follicular Tg on the Slc26a7 mRNA level using real-time PCR. FRTL-5 cells were exposed to increasing concentrations of Tg (0.1, 1, 5, and 10 mg/mL) for 24 h before mRNA purification. Real-time PCR revealed that Tg significantly suppressed the Slc26a7 mRNA level in a dose-dependent manner (Fig. 1A). When FRTL-5 cells were exposed to 5 mg/mL Tg for up to 24 h, Tg significantly suppressed the Slc26a7 mRNA level in a time-dependent manner (Fig. 1B).

We then examined the effect of Tg on SLC26A7 protein expression using Western blot analysis. Similar to the changes in the mRNA level, Tg suppressed the
SLC26A7 protein level in a dose- and time-dependent manner (Fig. 2A and 2B, upper panels). Densitometric analysis of the specific bands demonstrated suppression of the SLC26A7 protein level by Tg relative to the β-actin level (Fig. 2A and 2B, lower panels). These results indicate that follicular Tg suppresses SLC26A7 mRNA and protein levels in rat thyroid FRTL-5 cells, which is similar to the effect of TSH that we recently reported [12]. These results were somewhat surprising to us because Tg counteracted the action of TSH to modulate the mRNA and protein levels of most thyroid-specific genes, as described in the Introduction [6, 15, 16, 18-24].

**Tg suppressed SLC26A7 promoter activity**

We performed reporter gene assays to assess the promoter activity of SLC26A7 using luciferase reporter gene constructs that we created in our previous study [12]. FRTL-5 cells were treated with Tg 6 h after transfection of SLC26A7 promoter/luciferase chimeric plasmids, and the cells were maintained for 48 h. The results from luciferase reporter gene assay of four different SLC26A7 promoter regions showed that the activity of the 5'-2,207 SLC26A7 promoter was significantly suppressed by Tg (Fig. 3). The luciferase reporter gene activity was minimum in the constructs shorter than 5'-1,653, as demonstrated in our previous report [12]. This suggests that the observed decrease in the Slc26a7 mRNA level was the result of suppressed promoter activity.

**Tg and TSH additively suppressed the Slc26a7 mRNA level in FRTL-5 cells**

Since Tg and TSH independently suppressed the Slc26a7 mRNA level, we investigated the correlation between Tg and TSH to regulate Slc26a7 expression. We therefore examined the effect of Tg under lower concentrations of TSH (0.01 and 0.1 mU/mL) rather than the
maximal concentration used to maintain FRTL-5 cells (1 mU/mL). Real-time PCR showed that TSH at lower concentrations suppressed the Slc26a7 mRNA level as demonstrated previously [12] (Fig. 4, black bars). When Tg was added under lower concentrations of TSH, the Slc26a7 mRNA level was further suppressed in a dose-dependent manner, suggesting that the effect of TSH and Tg on suppression of the Slc26a7 mRNA level is additive (Fig. 4).

**Tg abolished the TSH-induced plasma membrane localization of SLC26A7 in FRTL-5 cells**

Localization to the plasma membrane is essential for SLC26A7 to function as an iodide transporter [8], and we previously reported that TSH is essential for inducing plasma membrane localization of SLC26A7 [12]. Therefore, we evaluated the potential effect of follicular Tg on TSH-induced SLC26A7 localization in FRTL-5 cells by immunofluorescence staining. FRTL-5 cells maintained without TSH were treated with 5 mg/mL Tg and incubated for 48 h. Immunofluorescence staining of SLC26A7 was performed, and its subcellular localization was observed by confocal microscopy. As a result, staining of SLC26A7 was predominantly observed in the cytoplasm after 48 h (Fig. 5, TSH + Tg). Thus, follicular Tg not only decreased SLC26A7 expression but also prevented its membrane localization, thereby disabling its transporter function.
SLC26A7 expression was inversely correlated with the level of Tg that accumulated in rat thyroid follicles

In a previous study using rat thyroid tissue sections, the level of radioiodine uptake in individual follicles was inversely correlated with the level of Tg that accumulated in follicles, reflecting suppression of iodide uptake by follicular Tg [19]. Even within the same follicle, radioiodine uptake was weaker in areas of dense Tg staining [19]. We therefore investigated the correlation between the Tg level and SLC26A7 expression in the follicle by double immunofluorescence staining of rat thyroid tissue sections. SLC26A7 was strongly expressed on the apical membrane where Tg accumulation was poor (Fig. 6, arrows), whereas it was barely observed where Tg accumulation was abundant (Fig. 6, arrowheads). In addition, even within the same follicle, accumulation of Tg and SLC26A7 localization on the apical membrane exhibited an inverse correlation (Fig. 6, upper left follicle).

Taken together, these data suggest that follicular Tg suppresses both the expression level and membrane localization of SLC26A7, thereby reducing its iodide transporter activity.

Discussion

In this study, we examined the effect of follicular Tg on the expression and subcellular localization of the novel apical iodide transporter SLC26A7. We showed that Tg at follicular concentrations suppresses both the mRNA and protein expression of SLC26A7 at the transcriptional level. In addition, we showed that Tg inhibited the ability of TSH to induce membrane localization of SLC26A7 both in vitro and in vivo, suggesting that follicular Tg reduces the iodide transport activity of SLC26A7, which in turn suppresses TH synthesis.

Dietary iodide is actively concentrated in thyrocytes via SLC5A5 and is secreted into the follicular lumen via SLC26A4 and SLC26A7, constituting the essential steps of TH biosynthesis. The expression, plasma membrane localization, and iodide transport activity of SLC5A5, a basolateral iodide transporter essential for iodide uptake, are tightly controlled by TSH. Thus, TSH induces the transcription, mRNA expression, protein expression, and plasma membrane localization of SLC5A5 [1, 28, 29]. In contrast, the expression levels of SLC26A4 and SLC26A7, apical iodide transporters that secrete accumulated iodide into the follicular lumen, are rather suppressed by TSH [12]. Interestingly, however, TSH increases the levels of SLC26A4 and SLC26A7 at the membrane, where they function as iodide transporters [12, 30]. Therefore, it is thought that TSH enhances the activity of apical transporters, although the mechanism underlying the suppression of protein levels is not known.

Although the action of TSH on these three iodide transporters is somewhat complex, as described above, the action of follicular Tg and its significance have been clarified. In previous studies, we demonstrated that follicular Tg significantly suppressed the expression of SLC5A5 and SLC26A4 and almost completely abolished the radioiodine uptake of SLC5A5 [16, 19, 21]. In the present study, we additionally demonstrated that follicular
Tg suppresses SLC26A7 expression and prevents its plasma membrane localization. These results are consistent with our studies showing that the function of individual follicles is tightly regulated by the level of Tg, a precursor of THs, that has accumulated in the respective follicular lumen. Thus, the activities of the follicle, e.g., the Tg content, iodide uptake, and TH accumulation in each follicle, are not uniform but rather quite heterogeneous [15, 19, 31-33]. Iodide uptake, Tg secretion, and TH synthesis actively take place in follicles with low Tg content. However, once enough TH precursor, i.e., Tg, has accumulated within a follicle, these processes are shut down by the autocrine negative-feedback action of follicular Tg, and the highly accumulated follicular Tg is ready for endocytosis by thyrocytes to secrete THs [16, 18, 23, 24]. The suppressed mRNA expression of both basolateral and apical iodide transporters and their dissociation from the plasma membrane by follicular Tg is considered part of the autocrine regulation of follicular function [16, 18, 23, 24].

The effect of Tg was also confirmed in rat thyroid tissue sections. We showed that the apical membrane localization of SLC26A7 was inversely correlated with the accumulation of follicular Tg. In our previous study using rat thyroid tissues following intraperitoneal injection of $^{125}$I, accumulation of $^{125}$I in the thyroid follicle demonstrated by tissue section autoradiography was also inversely correlated with Tg, according to immunohistochemistry [19]. These observations confirm the in vitro evidence that follicular Tg suppresses the gene expression and function necessary for TH synthesis.

We demonstrated that both Tg and TSH act on the same cis-element of the SLC26A7 promoter, in which three TTF-1 binding sites were found by in silico analysis [12]. Since TTF-1 expression is suppressed by TSH or Tg [15, 34], it is likely that TTF-1 is the key transcription factor regulating SLC26A7 promoter activity. In addition, it was reported that the transcriptional activity of SLC26A4 was suppressed by TTF-1 knockdown [35]. Therefore, two apical iodide transporters might be similarly regulated by TTF-1. To demonstrate the role of TTF-1 on SLC26A7 promoter activity, we are currently performing an in-depth study using electrophoretic mobility shift assay (EMSA).

The SLC26A family is predicted to contain an anti-sigma factor antagonist (STAS) domain within the COOH terminus that may be involved in plasma membrane localization and protein function [36]. A predicted phosphorylation site for protein kinase A (PKA) exists in the STAS domain of SLC26A4, and deletion of this site resulted in loss of plasma membrane localization and suppression of iodide transportation [30, 37]. SLC26A7 also contains a STAS domain at amino acid positions 495–631, and a mutation in this domain (p. Gln500Ter) induces SLC26A7 aggregation in the cytosol and suppression of its plasma membrane localization [8]. Since follicular Tg prevented TSH-induced plasma membrane localization of SLC26A7, Tg might interfere with the
PKA signaling pathway in FRTL-5 cells. Since protein kinase C (PKC) signaling antagonizes the effects of TSH [38], and Tg induces phosphorylation of MEK1/2 and ERK1/2 [39], which are downstream of the PKC/mitogen-activated protein kinase (PKC-MAPK) pathway [40], PKC signaling might also be involved in Tg actions. Further investigation is needed to determine the entire signaling pathway involved in Tg suppression of TSH-induced SLC26A7 expression and plasma membrane localization.

In summary, we have demonstrated that follicular Tg suppresses the expression and membrane localization of the novel apical iodide transporter SLC26A7, in addition to its previously known suppression of other iodide transporters (SLC5A5 and SLC26A4). It is still not understood how Tg, a huge macromolecule, is recognized by thyrocytes and how it exerts such a potent effect on gene expression regulation. Nevertheless, it is thought that modulating the signaling molecules essential for Tg action will enable regulation of iodide transport activity in thyrocytes, providing a novel therapeutic modality to control TH synthesis without affecting TSH function.

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Disclosure

None of the authors have any potential conflicts of interest in relation to this research.

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