Regulation of solute carrier family 26 member 7 (Slc26a7) by thyroid stimulating hormone in thyrocytes

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Abstract. Iodine transportation is an important step in thyroid hormone biosynthesis. Uptake of iodine into the thyroid follicle is mediated mainly by the basolateral sodium–iodide symporter (NIS or solute carrier family 5 member 5: SLC5A5), and iodine efflux across the apical membrane into the follicular lumen is mediated by pendrin (SLC26A4). In addition to these transporters, SLC26A7, which has recently been identified as a causative gene for congenital hypothyroidism, was found to encode a novel apical iodine transporter in the thyroid. Although SLC5A5 and SLC26A4 have been well-characterized, little is known about SLC26A7, including its regulation by TSH, the central hormone regulator of thyroid function. Using rat thyroid FRTL-5 cells, we showed that the mRNA levels of Slc26a7 and Slc26a4, two apical iodine transporters responsible for iodine efflux, were suppressed by TSH, whereas the mRNA level of Slc5a5 was induced. Forskolin and dibutyryl cAMP (dbcAMP) had the same effect as that of TSH on the mRNA levels of these transporters. TSH, forskolin and dbcAMP also had suppressive effects on SLC26A7 promoter activity, as assessed by luciferase reporter gene assays, and protein levels, as determined by Western blot analysis. TSH, forskolin and dbcAMP also induced strong localization of Slc26a7 to the cell membrane according to immunofluorescence staining and confocal laser scanning microscopy. Together, these results suggest that TSH suppresses the expression level of Slc26a7 but induces its accumulation at the cell membrane, where it functions as an iodine transporter.

Key words: SLC26A7, SLC5A5, SLC26A4, TSH, Thyroid

IODINE is a fundamental element for the biosynthesis of the thyroid hormones T3 and T4, and the prime function of the thyroid is to concentrate iodine and make it available for the biosynthesis of thyroid hormones. Iodine derived from dietary sources is transported into the thyrocyte across the basal membrane and then secreted across the apical membrane into the follicular lumen where it is organified to the tyrosine residues of thyroglobulin, a large protein stored in the follicular colloid. Thus, the thyroid follicle serves as the minimal functional unit of hormone synthesis and secretion.

Sodium–iodide symporter (NIS or solute carrier family 5 member 5: SLC5A5), the first iodine transporter cloned, in 1996 [1], is localized on the basolateral membrane of thyrocytes and is responsible for taking up circulating iodine into thyrocytes. SLC26A4 (or pendrin), another iodine transporter, was identified as a causative gene for Pendred syndrome in 1997, and the protein localizes on the apical membrane, opposite to SLC5A5 [2-4]. SLC26A7, encoding another member of the same transporter family as SLC26A4, was demonstrated to be a novel causative gene for congenital hypothyroidism in 2018 [5-7]. Although SLC26A7 was first identified as a chloride–bicarbonate anion exchanger in the kidney and stomach [8], expression profiling of different human tissues showed predominant mRNA expression of SLC26A7 in the thyroid. Immunofluorescence staining of thyroid tissue sections showed that SLC26A7 is localized at the apical membrane, similar to pendrin [3, 5, 9]. Bicameral culture system and radioiodine transport/uptake studies suggested that SLC26A7 functions in the efflux of iodine in culture medium [5], similar to pendrin [10]. SLC26A7 was thus considered a novel apical iodine transporter responsible for iodine efflux in the thyroid.

TSH is the primary regulator of iodine uptake and stimulator of thyroid hormone production and secretion.
TSH activates the Slc5a5 promoter to induce the expression and membrane localization of the protein [9, 11-14]. It also induced the membrane abundance of SLC26A4 and enhanced its function of iodine efflux into the follicular lumen, without affecting the total protein level [15, 16]. Although, the effects of TSH on SLC5A5 and SLC26A4 have been evaluated extensively, the role of TSH in the regulation of the newly identified SLC26A7 is not known. Here, we investigated the potential effect of TSH on Slc26a7 with respect to its expression and subcellular localization in rat thyroid FRTL-5 cells.

Materials and Methods

Cell culture and treatment

Rat thyroid FRTL-5 cells were grown in Coon’s modified Ham’s F-12 medium supplemented with 5% bovine serum (Invitrogen, Waltham, MA, USA) and a mixture of six hormones (1 mU/mL bovine TSH, 10 μg/mL insulin, 0.36 ng/mL hydrocortisone, 5 μg/mL transferrin, 10 ng/mL somatostatin and 2 ng/mL glycyrrhizin) as described previously [17-19]. After the cells attached to the bottom of plates (Greiner Bio-One, Kremsmünster, Austria), they were maintained in the same medium without TSH for 5 days, followed by exposure to TSH, forskolin or dibutyryl cAMP (dbcAMP, Sigma-Aldrich, St. Louis, MO, USA).

Total RNA isolation and real-time PCR

Total RNA was purified using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) as described previously [20-22]. Real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) according to the manufacturer’s instructions and run on the Thermal Cycler Dice Real Time System III (Takara Bio, Tokyo, Japan). Briefly, 5 ng cDNA was mixed with 10 μL 2× Fast SYBR Green Master Mix and amplified by incubating for 30 sec at 95°C, followed by 40 cycles of 5 sec at 95°C and 60 sec at 60°C, and 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 conducted in at least triplicate, and the relative mRNA expression levels were normalized to the corresponding Gapdh level using the ∆∆Ct method as described [19, 23, 24]. The sequences of the PCR primers are as follows: NM 052983.2 Slc5a5 forward, 5'-CTACCCGTGGGTGG TATGAAGG-3'; Slc5a5 reverse, 5'-TGCCACCCCACTAT GAAGTTCC-3'; NM_019214.1 Slc26a4 forward, 5'- CAAAGTGGAAGTCTTGGCTCCTCT-3'; Slc26a4 reverse, 5'-TTGGTGGGCTAGACCTTCTCCC-3'; XM_008763531.2 Slc26a7 forward, 5'-TGCTCCCAATGAACATCTCC-3'; Slc26a7 reverse, 5'-CAAAGCCACCTGTGCTTTTGCC-3'; NM_017008.4 Gapdh forward, 5'-ACAGCAACAGGGTG GTGGAC-3'; Gapdh reverse, 5'-TTTGAGGGTGAC CGAACTT-3'.

Protein preparation and Western blot analysis

Preparation of cellular proteins and Western blot analysis were performed as described previously [19, 22, 25]. Briefly, cells were washed with ice-cold PBS and lysed in lysis buffer 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 Na3VO4 and 1 μg/mL leupeptin. Cells were then scraped using a disposable cell lifter (Corning, Corning, NY, USA) and centrifugated. The supernatant was recovered, and protein concentrations were measured using the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Ten micrograms of each protein sample were separated by electrophoresis on NuPage 4–12% Bis-Tris gels (Invitrogen) and transferred to a nitrocellulose membrane using i-Blot gel 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, and then incubated with mouse anti-Slc26a7 (1:1,000; Novus Biologicals, Centennial, CO, USA) or mouse anti-β-actin (1:5,000; Cell Signaling Technology, Danvers, MA, USA) overnight. 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. The HRP was detected by chemiluminescence using the ImmunoStar LD reagents (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and analyzed by the C-DiGit blot scanner (both from LI-COR, Lincoln, NE, USA).

Luciferase reporter gene assay

Among four transcript variants of SLC26A7, we used one that has the most upstream transcription start site (NM 001282356.2). A series of sequences within the 5'-flanking region of the human SLC26A7 gene, starting at positions –2,207, –1,653, –1,140 and –443, relative to the transcription start site, were amplified by PCR from human genomic DNA using the following forward and reverse primer pairs containing NheI or XhoI restriction enzyme sites: –2,207 forward, 5'-CGTCTTAGCTGTTG CTTAGGGATC-3'; –1,653 forward, 5'-CGTCTAGCTA GGCTGTTATAACAAAGTAT-3'; –1,140 forward, 5'- CGTCTAGCTACTCTAGTGGAGGAGT-3'; –443 forward, 5'-CTAGCAGCAGCAGCAACTCC-3'; and reverse, 5'-GATCTCAGACTCCGTTACATCT CACGAC-3'. The PCR products were introduced into
the pGL3-Basic luciferase reporter plasmid (Promega, Madison, WI, USA) via the NheI and XhoI sites. FRTL-5

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(Thermo Fisher Scientific, Waltham, MA, USA) was added to the culture medium, and cells were maintained for 48 h. Cells were harvested, and a luciferase assay was performed using the Bright-Glo Luciferase Assay System (Promega). Luciferase activity was measured using the ARVO MX plate reader (PerkinElmer, Waltham, MA, USA) and normalized to the corresponding protein concentrations determined using the DC Protein Assay Kit (Bio-Rad Laboratories).

Immunofluorescence staining and confocal laser scanning microscopy

After attaching to the bottom of 4-well chamber slides (Thermo Fisher Scientific, Waltham, MA, USA), FRTL-5 cells were maintained in the absence of TSH for 5 days. Cells were then treated with 1 μM TSH for 6, 12, 24, 48 and 120 h, and 1 μM forskolin or 500 μM dbcAMP for 120 h. After incubation, 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 with PBS containing 3% bovine serum albumin, the slides were incubated with mouse anti- Slc26a7 (1:500; Novus Biologicals) overnight, washed with PBST and then incubated with Alexa Fluor 594-labeled goat anti-mouse IgG (1:1,000; Cell Signaling Technology), followed by nuclear staining with Hoechst 33342 (Cell Signaling Technology). Fluorescence was observed under a confocal laser scanning microscope (FV10i-DOC, Olympus, Tokyo, Japan).

Statistical analysis

All experiments were performed in triplicate and repeated at least three times. Results are expressed as the mean ± standard deviation (SD). Significant differences were determined by one-way ANOVA followed by Dunnett’s post hoc test. p < 0.05 was considered to represent significance.

Results

TSH suppressed Slc26a7 mRNA expression in FRTL-5 cells

We first examined the potential effect of TSH on the Slc26a7 mRNA level using real-time PCR. After culturing FRTL-5 cells in medium without TSH for 5 days, fresh medium supplemented with 0.01, 0.1 or 1 μM/mL TSH was added to the cells for up to 24 h. The real-time PCR results clearly showed that TSH significantly suppressed Slc26a7 mRNA levels in a concentration-dependent manner (Fig. 1A, left panel); on the other hand, TSH significantly induced Slc5a5 mRNA expression (Fig. 1A, middle panel), which has been demonstrated previously [12, 26]. The Slc26a4 mRNA level was also reduced by TSH (Fig. 1A, right panel), similar to the change in Slc26a7 mRNA. Such changes in mRNA levels were observed as early as 6 h after TSH stimulation and progressed until 24 h (Fig. 1B). These results suggest that TSH induces mRNA expression of Slc5a5, the basolateral iodine transporter responsible for iodine uptake, while TSH suppresses expression of the apical iodine transporters, Slc26a7 and Slc26a4, responsible for iodine efflux.

Forskolin and dbcAMP reproduced the suppressive effect of TSH on Slc26a7 mRNA expression

To further evaluate the observed action of TSH, we examined the effects of forskolin (which increases the intracellular cAMP concentration by stimulating adenylate cyclase [27]) and dbcAMP (a cell-permeable cAMP analog) on the Slc26a7 mRNA level. Real-time PCR revealed that both forskolin and dbcAMP suppressed the mRNA level of Slc26a7 in a concentration-dependent manner at 24 h (Fig. 2, left panels), which was similar to the effect of TSH, shown in Fig. 1. Forskolin and dbcAMP induced Slc5a5 mRNA expression (Fig. 2, middle panels) and reduced Slc26a4 mRNA expression (Fig. 2, right panels), also reproducing the effects of TSH. These results confirmed that the TSH/cAMP signaling cascade increases the expression of Slc5a5, encoding a basolateral iodine transporter, while the same signal suppressed the expression of Slc26a7 and Slc26a4, apical iodine transporters, in thyrocytes.

TSH suppressed Slc26a7 protein expression in FRTL-5 cells

We next examined the effect of TSH on Slc26a7 protein expression using Western blot analysis. FRTL-5 cells cultivated in the absence of TSH for 5 days were exposed to TSH, forskolin or dbcAMP at increasing concentrations for 24 h. Western blot analysis of the total cellular protein content clearly revealed that TSH, forskolin and dbcAMP all suppressed the Slc26a7 protein level in a concentration-dependent manner (Fig. 3, upper panels). Densitometric analysis of the specific bands confirmed the significant reductions in the Slc26a7 protein level relative to the β-actin level by TSH, forskolin and dbcAMP (Fig. 3, lower panels).

TSH suppressed human SLC26A7 promoter activity

To investigate whether TSH/cAMP signaling directly
affects the transcription of \textit{SLC26A7}, we cloned 5'-flanking regions of human \textit{SLC26A7} and performed luciferase reporter gene assays to evaluate the promoter activity. FRTL-5 cells maintained in the absence of TSH for 5 days were transfected with \textit{SLC26A7} promoter/luciferase chimeric plasmids or the control plasmid for 6 h. Then, the culture medium was replaced with fresh medium supplemented with TSH, forskolin or dbcAMP, and the cells were cultured for 48 h before luciferase assays were performed. Among the four \textit{SLC26A7} promoter constructs, that harboring the sequence extending to position –2,207 of the 5'-flanking region showed strong basal promoter activity in the absence of TSH, which was almost comparable with the activity of the pGL3 Control plasmid driven by the strong SV40 promoter and enhancer (Fig. 4, control). Whereas the promoter activity of the pGL3 Control plasmid was enhanced by TSH and not significantly affected by forskolin or dbcAMP under the conditions tested, the activity of the \textit{SLC26A7} promoter was significantly suppressed by TSH, forskolin and dbcAMP. These results suggest that the observed decrease in the \textit{Slc26a7} mRNA level was a result of suppression of \textit{SLC26A7} promoter activity. No significant transcriptional activity was detected in the other luciferase reporter constructs under any of the conditions tested (Fig. 4).

These results led to speculation that the promoter sequence between positions –2,207 and –1,653 is necessary for TSH regulation of \textit{SLC26A7}. This, \textit{in silico} analysis was performed to predict potential transcription binding sites using PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) and CiiiDER (http://ciiider.com/). The results revealed that three NK2 homeobox 1 (NKX2-1; also known as thyroid transcription factor-1: TTF-1) binding sites are located in the 5'-flanking region of \textit{SLC26A7}, from positions –2,207 to –1,653 (–2,090 to –2,085, –2,077 to –2,072, –2,051 to –2,046). In addition, the 5'-flanking region contained apparent TATA and CpG box as biological signals of core promoter elements. Therefore, NKX2-1 might be responsible for mediating the effects of TSH/cAMP signaling on \textit{SLC26A7} promoter activation, which is similar to the transcriptional regulation of other thyroid-specific genes such as \textit{Tg}, \textit{TPO}, \textit{SLC5A5} and \textit{TSHR} [14, 28, 29].

**TSH induced membrane localization of \textit{Slc26a7} in FRTL-5 cells**

As an apical iodine transporter, cell membrane localization is expected to be crucial for the function of
Slc26a7 [5]. To demonstrate the potential effects of TSH on the subcellular localization of Slc26a7, we analyzed Slc26a7 localization in FRTL-5 cells by immunofluorescence staining and confocal laser scanning microscopy. In basal medium without TSH, Slc26a7 protein was observed in the cytoplasm in a somewhat granular pattern especially around the nucleus (Fig. 5, 0 h). TSH stimulation resulted in Slc26a7 moving from the perinuclear area to the cytoplasm (Fig. 5, 6–12 h) and then gradually moving to the cell membrane (Fig. 5, 24–120 h). The fluorescence intensity of Slc26a7 in cells was reduced by TSH with time, which is in agreement with the decrease in the total Slc26a7 protein level demonstrated by Western blot analysis, shown in Fig. 3.

Fig. 2 Both forskolin and dbcAMP suppress Slc26a7 mRNA expression in a concentration-dependent manner in FRTL-5 cells
FRTL-5 cells were maintained in basal medium without TSH for 5 days and then switched to medium containing increasing concentrations of forskolin (0.01, 0.1 and 1 μM) or dbcAMP (50, 200 and 500 μM) for 24 h. Total RNA was extracted and subjected to real-time PCR to determine the relative mRNA levels of Slc26a7, Slc5a5, Slc26a4 and Gapdh. Relative mRNA levels were normalized to the Gapdh level and expressed relative to the control level (n = 3). * p < 0.05; ** p < 0.01; *** p < 0.001, compared with the control.

Fig. 3 TSH, forskolin and dbcAMP suppress Slc26a7 protein expression in a concentration-dependent manner in FRTL-5 cells
FRTL-5 cells were maintained in basal medium without TSH for 5 days and then switched to medium containing increasing concentrations of TSH (0.01, 0.1 and 1 mU/mL), forskolin (0.01, 0.1 and 1 μM) or dbcAMP (50, 200 and 500 μM) for 24 h. Total cellular proteins were extracted and subjected to Western blot analysis to determine the protein levels of Slc26a7 and β-actin (upper panels). The densitometric values of the specific bands are presented as bar graphs and represent the relative protein levels normalized to the β-actin level (lower panels).
Furthermore, both forskolin and dbcAMP exerted the similar effect as TSH to translocate Slc26a7 protein on the cell membrane (Fig. 5). Together, these results suggest that TSH/cAMP signaling induces translocation of the Slc26a7 protein to the plasma membrane, where it functions as a transporter, while suppressing its total cellular expression level.

Discussion

The present study aimed to elucidate the effects of TSH on the expression and subcellular localization of Slc26a7, a newly identified apical iodine transporter, in thyrocytes. We showed that stimulation of FRTL-5 cells with TSH, forskolin or dbcAMP suppressed Slc26a7 mRNA expression, as well as the activity of the SLC26A7 promoter, which contains three putative NKX2-1 binding sites. It was previously shown that the transcription of Slc26a4 was positively regulated by Nkx2-1 [30], and that TSH decreased Nkx2-1 protein expression as well as its association with the promoters of its target genes in FRTL-5 cells [31]. Therefore, the suppressive effect of TSH on the SLC26A7 promoter may be, at least in part, mediated by down-regulation of NKX2-1 expression via TSH. Detailed promoter analysis is needed to elucidate the whole picture of transcriptional regulation of SLC26A7 gene.

While suppressing the total cellular protein level of Slc26a7, TSH/cAMP exerted a completely different effect to concentrate Slc26a7 on the plasma membrane. When cells were maintained in TSH-free culture medium, the Slc26a7 protein was localized mainly around nuclei. After stimulation with TSH, Slc26a7 gradually translocated into the cytoplasm and then to the plasma membrane, as demonstrated by confocal laser scanning microscopy. Interestingly, this is similar to the observed changes in Slc5a5 localization induced by TSH, in which Slc5a5 localized to intracellular compartments throughout the cytoplasm in the absence of TSH but localized to the plasma membrane in the presence of TSH [12]. Regarding SLC26A4, we previously showed that SLC26A4 proteins harboring mutations (L236P, T416P and G384E), identified in patients with Pendred syndrome exhibiting defective iodine organification,

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**Fig. 4** TSH, forskolin and dbcAMP suppress human SLC26A7 promoter activity

(A) FRTL-5 cells maintained in basal medium without TSH were transfected with the pGL3-Basic luciferase reporter plasmid carrying 5'-flanking regions of human SLC26A7 of the indicated lengths, or with the pGL3 Control plasmid. The cells were then treated with 1 mU/mL TSH, 1 μM forskolin or 500 μM dbcAMP for 48 h. Cells were harvested, and luciferase assays were performed. Promoter activity is expressed as mean ± SD relative light units (n = 3). All values were normalized to the total amount of cellular protein. (B) Schematic representation of SLC26A7 promoter region. Nkx2-1 binding site, GC box, CAAT box and TATA box are shown relative to the transcription start site (TSS).
were retained in the endoplasmic reticulum and not seen at the plasma membrane [32]. Similarly, in vitro studies showed that over-expressed FLAG-conjugated wildtype SLC26A7 was detected mainly at the cell membrane, where it exported intracellular iodine outside of the cell, whereas a mutant SLC26A7 protein aggregated in the cytosol instead of localizing to the cell membrane and no longer exhibited iodine transporter function [5]. Taken together, the migration of SLC26A7 from the cytoplasm to the plasma membrane induced by TSH suggests activation of its transporter function. Future electrophysiological studies are needed to determine whether TSH actually enhances the transporter function of SLC26A7, along with its membrane localization.

We showed a similarity between the regulation of Slc26a7 and Slc26a4, two apical iodine transporters, by TSH. Previous studies showed that TSH suppressed the gene expression and induced membrane localization of Slc26a4 [15, 16], as observed with Slc26a7 in this study. In rat thyroid PCCL-3 cells, TSH and forskolin rapidly increased Slc26a4 abundance at the plasma membrane via the TSH/cAMP/PKA signaling cascade [16], which was correlated with an increase in iodine efflux [16]. It is not clear why the expression levels of the apical iodine transporters Slc26a7 and Slc26a4 are decreased, but their membrane localization is induced, by TSH. Since FRTL-5 cells are monolayer cells without having polarization, studies of the effect of iodine on Slc26a7 expression using polarized cells may provide a clearer picture for understanding the fine-tuning of iodine-transporting mechanisms.

Belonging to the same transporter family, Slc26a7 and Slc26a4 share a common protein structure. Two-thirds of the NH2 terminus is largely hydrophobic and encompasses the transmembrane segments, in which the anion-binding sites and pores are putatively located. The COOH-terminal domain of the protein is predicted to contain a sulfate transporter and anti-sigma factor antagonist (STAS) domain [33]. Studies on Arabidopsis thaliana SLC26 transporters and disease-causing mutations in the STAS domain of SLC26A3 suggest that this domain is important for the membrane anchoring and transporter function of SLC26 family members [34]. Elimination of the distal portion of the STAS domain containing a putative PKA phosphorylation site (RKDT714–717) in SLC26A4 resulted in partial loss of function and decreased membrane localization in response to TSH or forskolin [15, 16]. Further, in Japanese patients with goitrous congenital hypothyroidism, a p.Gln500Ter mutation was identified in the STAS domain of SLC26A7 [5], and expression of this mutant protein in HEK293T cells caused abnormal localization in the cytosol and loss of iodine transporter function [5].
Taken together, our findings and those from other groups suggest that the TSH signaling cascade may induce Slc26a7 membrane localization via phosphorylation of the STAS domain of Slc26a7. This post-transcriptional modification, in addition to transcriptional regulation, is an important way for TSH to regulate the expression level of Slc26a7 in FRTL-5 cells. However, TSH induced membrane localization of Slc26a7, suggesting that the transporter function is activated by TSH. These results encourage further studies to explore the underlying mechanisms by which TSH regulates the subcellular localization along with the transporter function of Slc26a7.

Acknowledgements

The authors would like to thank Ms. Madoka Naito, Teikyo University for her secretarial assistance. This work was supported by JSPS KAKENHI Grant Number JP15K09444, JP17K08990 and JP19K07875.

Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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