De novo triiodothyronine formation from thyrocytes activated by thyroid-stimulating hormone

The thyroid gland secretes primarily tetraiodothyronine (T₄), and some triiodothyronine (T₃). Under normal physiological circumstances, only one-fifth of circulating T₃ is directly released by the thyroid, but in states of hyperactivation of thyroid-stimulating hormone receptors (TSHRs), patients develop a syndrome of relative T₃ toxicity. Thyroidal T₃ production results from iodination of thyroglobulin (TG) at residues Tyr⁵ and Tyr¹⁰⁳, whereas thyroidal T₃ production may originate in several different ways. In this study, the data demonstrate that within the carboxyl-terminal portion of mouse TG, T₃ is formed de novo independently of deiodination from T₄. We found that upon iodination in vitro, de novo T₃ formation in TG was decreased in mice lacking TSHRs. Conversely, de novo T₃ that can be formed upon iodination of TG secreted from PCCL3 (rat thyrocyte) cells was augmented from cells previously exposed to increased TSH, a TSHR agonist, a cAMP analog, or a TSHR-stimulating antibody. We present data suggesting that TSH-stimulated TG phosphorylation contributes to enhanced de novo T₃ formation. These effects were reversed within a few days after removal of the hyperstimulating conditions. Indeed, direct exposure of PCCL3 cells to human serum from two patients with Graves’ disease, but not control sera, led to secretion of TG with increased intrinsic ability to form T₃ upon in vitro iodination. Furthermore, TG secreted from human thyrocyte cultures hyperstimulated with TSH also showed an increased intrinsic ability to form T₃. Our data support the hypothesis that TG processing in the secretory pathway of TSHR-hyperstimulated thyrocytes alters the structure of the iodination substrate in a way that enhances de novo T₃ formation, contributing to the relative T₃ toxicosis of Graves’ disease.

In the body of vertebrate animals, thyroglobulin (TG) is the primary (if not exclusive) original source of thyroid hormones (1) that regulate central nervous system development and function, oxidative metabolism, thermogenesis, and body weight regulation, heart rate, cardiac output, LDL cholesterol levels, and other phenotypes (2, 3). The thyroid gland produces virtually 100% of the supply of L-thyroxine (T₄) from the body. However, other than nongenomic actions (4), the main physiological effects of thyroid hormones are brought about by gene expression changes as a consequence of 3,3',5-triiodothyronine (T₃) interaction with nuclear thyroid hormone receptors (5).

Depending upon the species and conditions, there are somewhat differing views about the main sources of circulating T₃. In otherwise normal thyroidectomized rats that are fully replaced with exogenous levothyroxine (i.e. normal serum T₄), circulating T₃ is decreased ~55% (6) indicating a significant thyroidal contribution to circulating T₃. In normal humans, classic studies have estimated that only ~21% of daily T₃ production is derived from thyroidal secretion (the rest coming from deiodination of T₄ to T₃ by deiodinases D1 and D2) (7). However, in patients with untreated Graves’ disease (a disease of thyroidal hyperstimulation by TSH receptor-stimulating antibodies (8)), thyroid tissue is markedly enriched in T₃ concurrent with increased T₃ in the circulation (9, 10). Although some increased thyroidal T₃ production in Graves’ disease might be derived from intrathyroidal deiodination of T₄ to T₃ (11), the aforementioned study of untreated Graves’ patients reported increased thyroid tissue T₃ only after Pronase digestion (7). Moreover, mice with whole body D1/D2-double knock-out (DKO) nevertheless maintain normal circulating T₃ levels (12).}

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2 The abbreviations used are: TG, thyroglobulin; PTU, propylthiouracil; T₄, triiodothyronine; T₃, thyroxine; TSH, thyroid stimulating hormone; TSHR, TSH receptor; D₁, type 1 deiodinase; D₂ type 2 deiodinase; DKO, D₁/D₂ double knockout; DIT, diiodotyrosine; MIT, monoiodotyrosine; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; qPCR, quantitative PCR.
together, these findings strongly imply that the thyroid gland has the capability to contribute importantly to circulating T₃ via a mechanism involving de novo T₃ formation, and this may be particularly important in Graves’ disease.

The role of TG (a large homodimeric glycoprotein with a monomer molecular mass of 330 kDa and containing >2745 residues) in thyroid hormone synthesis is initiated upon its iodination (13, 14). Iodination is catalyzed by thyroid peroxidase, which provides the necessary oxidation to form diiodotyrosine (DIT) and monoiodotyrosine (MIT) within TG. Favored by these same oxidizing conditions, a coupling reaction involving a DIT acceptor residue and a corresponding DIT donor residue allows for the formation of T₄ within the TG polypeptide; similarly, coupling of an MIT donor with a DIT acceptor allows for de novo T₃ formation (15, 16). Classic studies report that thyroid peroxidase shows no marked specificity in its ability to catalyze TG iodination and coupling over that of lactoperoxidase or myeloperoxidase (16), whereas efficient T₄ and T₃ formation requires the TG substrate in its native conformation (17). Furthermore, despite ~70 Tyr residues distributed broadly along the length of the protein, T₄ and T₃ formation are restricted to relatively few sites in TG, including an evolutionarily preferred DIT–DIT coupling of Tyr¹³⁰–Tyr⁵ to yield T₄ at position 5 (1) and a preferred T₃ formation site at position 2746 of human TG (2744 of mouse TG, although the MIT coupling partner in either species remains unclear) (18, 19).

During its complex trafficking through the intracellular transport pathway of thyrocytes, TG undergoes considerable post-translational processing prior to its secretion and iodination (20). Many of these post-translational modifications are regulated indirectly by TSH-induced changes in the gene expression and activity of TG processing enzymes (21). Herein, we have examined de novo T₃ formation within TG analyzed both from in vivo samples and after iodination in vitro. We directly demonstrate de novo T₃ formation in TG and establish that this ability is directly related to the degree to which thyrocytes have been exposed to prior TSHR stimulation.

Results

De novo formation of T₃ within TG

We developed a simple assay to detect the presence of T₃ formation within thyroidal protein of euthyroid mice by immunoblotting using a mAb that recognizes T₃ when contained within the TG protein backbone, in parallel with immunoblotting with a polyclonal antibody against TG. The addition of free T₃ (half-maximal concentration ~75 ng/ml) eliminated the immunoblotted mouse TG band with mAb anti-T₃, whereas the addition of free T₄ had little effect (Fig. 1A, blots at left; quantitation at right). Thyroidal immunofluorescence from euthyroid mice with mAb anti-T₃ was distributed primarily in the follicle lumen where extracellular TG resides in “colloid”; this immunofluorescent signal was fully blocked by the addition of free T₃ at 500 ng/ml but was only slightly diminished by free T₄ even at 2000 ng/ml (Fig. 1B). Routinely, we added 500 ng/ml of free T₃ in all immunoblotting experiments designed to detect T₃ in TG, to ensure specificity. The data in Fig. 1 indicate that a basal level of T₃ in TG is present under euthyroid (i.e. not TSH-hyperstimulated) conditions.

The primary sequence of mouse TG encodes a single predicted site for Factor Xa cleavage that excises the N-terminal one-third of the protein from the C-terminal two-thirds. As Dunn et al. (18) reported that the majority of T₃ synthesized within TG is located in its C-terminal portion, we extracted mouse thyroid tissue and found by immunoblotting that a single band of intact TG was cleaved to two fragments of the expected molecular mass after incubation with Factor Xa (Fig. 1C, left). Of these, the larger fragment corresponding to the C-terminal portion of TG was selectively enriched in T₃ (Fig. 1C, right).

There are strong cell biological arguments to suggest that T₃ contained within the TG protein does not result as a consequence of deiodination of T₄ contained within TG. Specifically, almost all of the hormone-containing-TG is localized to the extracellular thyroid follicular lumen (Fig. 1B) (22), whereas the catalytic activities of the two enzymes responsible for T₄ to T₃ conversion (deiodinases D1 and D2) are topologically facing the cytosol (23). Indeed, we examined the T₃ content of TG in the thyroids of animals devoid of D1 and D2 (12) and found that D1/D2-DKO mice had as much or more T₃ contained within TG than in that from a wild-type reference animal (Fig. 2, left). All of the detected signal was derived from bona fide protein-bound T₃ as it was completely blocked by the addition of free T₃ added to the immunoblotting conditions (Fig. 2, right). As a negative control, TG obtained from PTU-treated mice contained no detectable T₃ (Fig. 2 left). Together, these data provide strong support that de novo T₃ formation within TG is independent of T₄ deiodination.

TG secreted from thyrocytes after stimulation of TSHR is intrinsically more competent for de novo T₃ formation

Iodination of TG is enhanced by TSH stimulation of the thyroid gland (21). However, independent of effects on sodium iodide symport activity, dual-function oxidase activity, or thyroid peroxidase activity, it has been hypothesized that the intrinsic ability of TG to form T₃ may be modulated through prior thyroidal stimulation by TSH on its receptor (thyroid-stimulating hormone receptor (TSHR) (24)). To test this, we subjected the TG obtained from thyroids of TSHR-KO mice, and from PTU-treated WT mice (that develop hypothyroidism and a compensatory increase in TSH leading to stimulated TSHRs) to iodination under fixed conditions in vitro. In addition to eliminating any effects of TSHR stimulation on thyrocyte enzymes that promote TG iodination in vivo, the in vitro iodination method also normalizes for TG protein, and total protein, within each iodination reaction. Prior to the in vitro iodination reaction, neither thyroids from TSHR-KO nor PTU-treated mice exhibited detectable T₃ within TG, as judged both by mAb anti-T₃ immunofluorescence and immunoblotting (Fig. 3, A and B). This is precisely why, for this experiment, we did not examine TG from control mouse thyroid glands. After iodination in vitro, TG from TSHR-KO consistently exhibited less de novo T₃ formation within TG (Fig. 3B, quantified in C).

To directly test the effect of thyrocyte stimulation by TSH on the ability of TG to facilitate de novo T₃ formation, we collected...
secretion from the rat thyrocyte cell line, PCCL3. Ordinarily, these cells are grown in the presence of TSH at a concentration of 1–10 milliunits/ml (25, 26). Under standard cell culture conditions, PCCL3 cells cannot iodinate their own secreted proteins; however, upon collection of the secretion followed by iodination \textit{in vitro}, de novo T3 formation was readily detectable within TG (Fig. 4A). We cultured PCCL3 cells for several days in two concentrations of TSH that differed by an order of magnitude (10 and 100 milliunits/liter). TG secreted from PCCL3 cells after exposure to the higher TSH concentration consistently exhibited greater potential for T3 formation upon iodination \textit{in vitro} (Fig. 4B, quantified in graph below the blot). Recently, a specific small molecule agonist of the TSHR, MS437, was described to activate G_{\alpha}\textsubscript{G}, which mimics TSH in turning on downstream target genes (27). Upon growth of PCCL3 cells in the presence of MS437, \textit{in vitro} iodination of
secreted TG led to a marked increase in de novo T₃ formation (Fig. 4C, quantified in graph above the blot). Stimulatory G proteins in thyrocytes activate adenylyl cyclase, which catalyzes cAMP synthesis (28). Upon incubation of PCCL3 cells in the presence of dibutyryl cAMP, in vitro iodination of secreted TG again demonstrated increased de novo T₃ formation (Fig. 4D, quantified in the graph above the blot). These data strongly suggest that TSH, working through TSHR, stimulatory G proteins, and cAMP production, alters the intrinsic ability of TG to form T₃ upon TG iodination.

There are several significant post-translational modifications that may have structural consequences on TG synthesized in TSH-stimulated thyrocytes, which may impact its hormono-genic potential, including de novo T₃ formation. These include the following: increased N-linked glycosylation (29) promoted in part by stimulation of oligosaccharyl transferase activity (30) and up-regulation of N-acetylglucosaminyltransferase 1 (31); increased complex sugars added in the thyrocyte Golgi complex including galactose (32); a marked decrease in the level of α2,6-bound sialic acid (33); down-regulation of tyrosine sulfation (34); increased formation of dityrosine cross-bridges (35) that can form non-disulfide-linked covalent TG dimers (36) and also increased TG phosphorylation (37), which can occur within TG carbohydrate, phosphotyrosine, and phosphoserine residues.

Recently, Fam20C was identified as a secretory pathway kinase that phosphorylates hundreds of secreted proteins, with a marked preference for Ser residues within the consensus sequence Ser-X-Glu/phospho-Ser (38). We observed a TSH dose-dependent 3-fold stimulation of Fam20C mRNA levels in PCCL3 cells (Fig. 5A). To examine the potential contribution of increased Fam20C mRNA on de novo T₃ formation in TG, we eliminated this increase using siRNA knockdown of Fam20C in TSH-stimulated PCCL3 cells (Fig. 5B). Upon in vitro iodination of secreted TG, TSH-stimulated cells with knockdown of Fam20C showed significantly less de novo T₃ formation (Fig. 5C, quantified in D). Moreover, treatment with calf intestinal phosphatase to dephosphorylate TG secreted from TSH-stimulated PCCL3 cells decreased de novo T₃ formation by 26% (data not shown). Taken together, these data suggest that TG phosphorylation (37) is one of the TSH-stimulated post-translational modifications that contribute to altering the structure of TG to increase de novo T₃ formation within TG.

Graves’ disease involves thyroidal overactivity leading to thyrotoxicosis, with a particular predilection to increased circulating T₃, i.e. relative T₃ toxicosis (9). To determine whether a TSHR-stimulating immunoglobulin could also promote enhanced de novo T₃ formation, PCCL3 cells were cultured for 5 days in the presence or absence of KSAb1, a strong TSHR-stimulating immunoglobulin (39). After the culture period, serum-free secretion from PCCL3 cells was collected for the ensuing 24 h and the secreted proteins were iodinated in vitro. From the results shown in Fig. 6A, left (quantified at right), it was clear that TG secreted from PCCL3 cells that had been previously incubated with KSAb1 showed significantly increased de novo T₃ formation.

Thyrotoxicosis and complications of Graves’ disease have been found to improve when the elevated concentrations of...
circulating TSHR-stimulating immunoglobulins are reversed, for example, by plasmapheresis (40). To examine reversibility of enhanced de novo T3 formation in TG, we washed out the KSAb1 and collected TG secreted over the next 24 h, or the subsequent 48 h thereafter. Upon iodination in vitro, it was apparent that beyond 24 h after washout of KSAb1, the subsequent secretion contained TG that had reverted back to a decreased ability for de novo T3 formation (Fig. 6B, left, quantified at right).

One clinical assay to screen for TSHR-stimulating immunoglobulins involves measuring responses to antibodies contained within the sera of human Graves’ patients, in a cultured rat thyrocyte cell line (41). With this in mind, we cultured PCCL3 cells for 5 days in the presence of control sera from 5 individuals without Graves’ disease or from 2 patients with Graves’ disease. After exposure to the human sera, serum-free secretion from PCCL3 cells was collected for the ensuing 24 h and the secreted proteins were iodinated in vitro. From the results shown in Fig. 7, culture with each of the 5 control sera resulted in subsequent TG secretion with approximately the same potential for de novo T3 formation. By contrast, culture with unpurified sera from two Graves’ disease patients both suggested an increase in the ability of the subsequently secreted TG to form T3 upon iodination.

Finally, we collected secretion from cultured primary human thyrocytes that were obtained from normal thyroid tissue of patients undergoing total thyroidectomy for localized thyroid cancer. In monolayer culture in TSH-containing medium to which additional iodide was not added, there was no detectable T3 found within TG secreted into the culture medium, regardless of the TSH dose (Fig. 8A). However, when the TSH concentration in the culture medium was increased by an order of magnitude, upon iodination in vitro, T3 formation in the subsequently secreted TG was clearly apparent (Fig. 8B). Taken together, these data indicate that both human TSHR-stimulating immunoglobulins, and human thyrocytes responding to stimulation of TSHR, increase de novo T3 formation in TG.

Discussion

Pioneering work of Dunn and others (15) helped lead to the identification of a few selected Tyr residues of the ~70 on the
TG protein that are favored for T₄ and T₃ formation. Remarkably, within the huge TG polypeptide, the most frequent site of T₄ formation resides just 5 residues from the N terminus (42), whereas more than half of all T₃ in TG is formed just 3 residues from the C terminus (24). It is known that DIT located at Tyr130 is the DIT “donor” to form T₄ at Tyr5, but the precise mechanism of T₃ formation at the C terminus has not been established (19), although it has been postulated to involve one of several potential upstream MIT donor residues in the TG polypeptide (43) including residue 2520 of human TG (44).

In this study, we have utilized a simple immunoblotting procedure to specifically identify T₃ within TG (Fig. 1). Essentially all of the immunodetectable T₃ in TG from mouse thyroid tissue (Fig. 1B) resides within the C-terminal portion of the TG polypeptide.  

**Figure 5. Fam20C expression regulated by TSH stimulation in PCCL3 thyrocytes.** A, PCCL3 cells were grown in complete media containing 10, 100, or 1000 milliunits/liter of TSH as described under “Experimental procedures.” At 48 h, cells were lysed, RNA was isolated and reverse transcribed, and Fam20C mRNA levels were quantified by qPCR normalized to that of HPRT1; the mean of 6 independent samples (each performed in duplicate) is shown; mean ± S.D.; *, p < 0.05. B, PCCL3 cells were lysed 48 h after siRNA-mediated knockdown of Fam20C or scrambled oligonucleotide control. The cells were grown in complete normal growth media (1000 milliunits/liter of TSH) prior to RNA isolation, and Fam20C mRNA was quantified by qPCR as in panel A (7 independent samples per condition). C, control secretion from PCCL3 cells (10 milliunits/liter of TSH) was collected as described in the legend to Fig. 4B; secretion from PCCL3 cells treated as in B was collected in serum-free cells in the presence of 1000 milliunits/liter of TSH. Secreted TG from all samples was iodinated in vitro followed by SDS-PAGE (3 μg of protein/lane), electrotransfer, and immunoblotting with mAb anti-T₃ and anti-TG, as indicated. D, quantitation of the relative T₃/TG band intensity ratio from 9 independent samples is shown; mean ± S.D.; *, p < 0.05 compared with scrambled oligonucleotide.

**Figure 6. Effects of TSHR-stimulating immunoglobulin (KSAb1) on de novo T₃ formation in TG.** A, left: PCCL3 cells were preincubated with mouse mAb KSAb1 TSHR-stimulating immunoglobulin (3 μg/ml). Secreted TG was iodinated in vitro followed by SDS-PAGE (3 μg of protein/lane), electrotransfer, and immunoblotting with mAb anti-T₃ and anti-TG, as indicated. Wild-type TG was run in an adjacent lane as a 330-kDa molecular mass marker. Right, quantitation of the relative T₃/TG band intensity ratio from 6 independent experiments is shown; mean ± S.D.; *, p < 0.05 compared with control lacking KSAb1 pretreatment. B, reversion of the stimulating effect of KSAb1. Left, the bathing media containing secreted TG was collected 1 day after removing KSAb1, and again for another 2 days after the first media collection. Secreted TG was iodinated in vitro followed by SDS-PAGE (2 μg of protein/lane), electrotransfer, and immunoblotting with mAb anti-T₃ and anti-TG, as indicated. Right, quantitation of the relative T₃/TG band intensity ratio from TG collected on days 2–3, compared with day 1 (normalized to 1.0) in 6 independent experiments is shown; mean ± S.D.; *, p < 0.05.
Figure 7. Stimulation of de novo T₃ formation by serum from two human Graves’ disease patients. A, PCCL3 cells were incubated for 5 days in media containing 40% serum from two patients with Graves’ disease (#6 and #7), or 5 euthyroid controls (#1–#5). After washing, TG secreted thereafter into serum-free media bathing the cells was iodinated in vitro and analyzed by SDS-PAGE (2 g of protein/lane), electrotransfer, and immunoblotting with anti-T₃ and anti-TG, as indicated. Wild-type TG was run in an adjacent lane as a 330-kDa molecular mass marker. B, quantitation of the relative T₃/TG band intensity with the ratio set to a value of 1.0 for control serum #1. For all sera tested, secreted TG was analyzed in at least three independent cultures and iodination in vitro; mean ± S.D.; *, p < 0.05 compared with controls.

Figure 8. De novo T₃ formation in TG secreted from primary culture of human thyrocytes. Human thyrocytes were grown at two different TSH concentrations: 100 or 1000 milliunits/ml, as described under “Experimental procedures.” A, TG secreted into serum-free media that was not iodinated in vitro served as a negative control; analyzed by SDS-PAGE (2 μg of protein/lane), electrotransfer, and immunoblotting with mAb anti-T₃ and anti-TG, as indicated. B, TG secreted from human thyrocytes grown as in panel A was iodinated in vitro and analyzed as in panel A.

molecule (Fig. 1C) and this immunoreactivity is independent of T₄ to T₃ conversion (Fig. 2). However, from TG secreted both in mouse thyroid glands (Fig. 3) and a (rat-derived) thyrocyte cell line (Fig. 4), the degree of prior stimulation of TSHRs exerts a strong influence over the efficiency of de novo T₃ formation upon TG iodination. Similar effects are observed either with high TSH itself (Fig. 4B), a TSHR agonist (Fig. 4C), a cAMP analog (Fig. 4D), or a TSHR-stimulating immunoglobulin (Fig. 6A). Moreover, these stimulating effects on de novo T₃ formation are entirely reversible within a day after removing the TSHR-stimulating immunoglobulin (Fig. 6B). Our data are consistent with the work of Fassler et al. (24), who proposed that TSH alters the utilization of hormonogenic sites on TG through changes in TG structure, which is presumed to be a consequence of altered TG post-translational processing.

Of the many potential TSH-stimulated changes in gene expression of TG post-translational processing enzymes, we have examined Fam20C, a novel secretory pathway kinase often referred to as “casein kinase” (38). Of the potential sites that are both predicted casein kinase (45) and canonical Fam20C targets, multiple sites are specifically conserved between rat, mouse, and human TG; three of these fall within the ChEl domain; and one of these (at position 2721 of human TG) has been directly established to be a phospho-Ser residue by mass spectrometry of human TG (46) and is close to a primary site of T₃ formation (24). Our current evidence suggests that TSHR stimulation can increase Fam20C mRNA levels within 48 h, and this increase contributes (along with other changes) to enhanced de novo T₃ formation within TG (Fig. 5).

The observation that TSHR stimulation up-regulates the efficiency of de novo T₃ formation upon TG iodination fits plausibly with the notion of a direct increase in de novo T₃ formation within TG in Graves’ disease, thereby contributing to increased intrathyroidal and secreted T₃ in this condition (7, 11), which contributes to a state of relative T₃ toxicosis (47, 48). Indeed, when collecting the secretion from PCCL3 cells incubated with serum from two Graves’ disease patients, the secreted TG showed a clearly increased predisposition to form T₃ upon iodination, in comparison to various control sera (Fig. 7). Moreover, TSHR activation also promotes enhancement of T₃ formation in TG from human thyrocytes, as directly demonstrated in cultures of normal human thyroid tissue hyperstimulated with TSH (Fig. 8).

Interestingly, from repeated experiments, we were unable to detect any demonstrable evidence that TSHR stimulation alters TG in a way that enhances its efficiency in de novo T₃ formation (data not shown), and this selectivity for de novo T₃ formation is consistent with previous reports (49). Of course, in Graves’ disease, there is also a general increase of T₄ secretion that may be attributed to many TSHR-stimulated activities including increased iodide uptake, DUOX function, TPO function, TG synthesis, and endocytosis of colloid, to name but a few (50). More work is needed at both the TG structural level, and at the thyroid cell biological level, to understand how and why T₃ is formed preferentially at residue 2746 of human TG (2744 of mouse TG), and how TSHR stimulation selectively increases de novo T₃ formation. However, our data support the hypothesis that the TSHR-stimulated effects reflect alterations in TG post-translational processing that impact structurally on the carboxyl-terminal region of TG molecules to enhance de novo T₃ formation.
Experimental procedures

Materials

Nal, lactoperoxidase, glucose oxidase, dextrose, fetal bovine serum, insulin, hydrocortisone, apo-transferrin, TSH, dibutyryl-cAMP, l-thyroxine (T₄), and 3,3',5-triiodo-l-thyronine (T₃) were from Sigma. Protease inhibitor mixture was from Roche Applied Science. Penicillin/streptomycin, PBS, and DMEM/F-12 were from Gibco. Prolong gold anti-fade reagent with DAPI, lithium dodecyl sulfate gel loading buffer, 4–12% polyacrylamide BisTris “NuPAGE” gels, 10% polyacrylamide Tris glycine gels, SeeBlue Plus2 molecular weight markers, and the SuperScript III first-strand synthesis system were from Invitrogen. BCA protein assay and RNAiMAX reagent were from Thermo Scientific; RNeasy RNA purification kit was from Qiagen; Factor Xa protease from New England Biolabs; and Citrisolv and Retrieve-All antigen retrieval reagent were from Fisher.

Antibodies

Mouse mAb anti-T₃ clone 3A6 (≤0.1% cross-reactivity with T₄ by ELISA), and a cross-adsorbed goat anti-mouse antibody conjugated to Alexa Fluor-555 were from Invitrogen; rabbit polyclonal anti-TG was as described (51). HRP-conjugated goat anti-mouse IgG was from Bio-Rad; HRP-conjugated goat anti-rabbit IgG was from Jackson ImmunoResearch.

Animal thyroid tissues used in this study

Mice were housed and fed as per an approved institutional protocol. C57BL/6 (WT) mice were 8–11 months old; D1/D2 double knock-out (DKO) mice (12) were 10 months old; TSHR-KO mice (52) were 3–5 months old. Where indicated, WT mice were fed low-iodide chow containing 0.15% PTU (Envigo) for 6–15 weeks. Thyroid glands were lysed by sonication in RIPA buffer ± SDS and containing a protease inhibitor mixture. Lysates were cleared at 12,000 × g for 10 min at 4 °C and total protein was determined by BCA or Bramhall assay (53).

Immunofluorescence with mAb anti-T₃

Fresh thyroid tissues from wild-type, PTU-treated, or TSHR-KO mice were immersion fixed with 10% formaldehyde and paraffin-embedded. Six-μm tissue sections were de-paraffinized with Citrosolv, followed by antigen retrieval with Retrieve-All, and permeabilization with Triton X-100 (0.2%). First antibody incubation with mAb anti-T₃ (1:200) in TBS plus 0.2% Tween 20 and 3% BSA ± free T₄ or free T₃ as described, was followed by a secondary antibody (noted above) diluted 1:5000 in the same buffer without hormone competitor. Images were captured using a ×40 oil objective in an Olympus Fluoview 500 laser scanning confocal microscope.

Site-specific protease digestion of TG

Factor Xa protease has only one predicted cleavage site (Peptide Cutter, Exasypt) in WT mouse TG, at amino acid 1036. Thyroid homogenate protein from C57BL/6 WT mice (140 μg) in RIPA buffer was adjusted to 1% SDS, 5 mM DTT and boiled for 1 min. The sample was then diluted to 0.01% SDS, 0.05 mM DTT in a total volume of 100 μl and digested with 8 μl of Factor Xa (New England Biolabs) for 15 min at 25 °C. Digestion was terminated by boiling in denaturing gel sample buffer containing 100 mM DTT for 5 min before SDS-PAGE.

PCCCL3 cell culture and treatments

PCCCL3 cells were cultured in DMEM/F-12 supplemented with 5% FBS plus penicillin/streptomycin and a four-hormone mixture containing 1 μg/ml of insulin, 1 nm hydrocortisone, 5 μg/ml of apo-transferrin, and 1 milliunit/ml of TSH.

For experiments with varying TSH concentrations, PCCCL3 cells were seeded at 50,000 cells/well in 24-well plates. After 24 h, the cells were grown for 5 days in complete medium containing TSH at either 10 or 100 milliunits/liter. The cells were then washed in PBS and re-fed at the same TSH concentrations in serum-free media and cultured for 1 (100 milliunits/liter of TSH) or 3 days (10 milliunits/liter of TSH), leading to comparable amounts of secreted TG and total protein in the bathing media.

For experiments studying the TSHR agonist MS437 (27), the PCCCL3 cells were seeded as above and grown for 3 days in complete medium with 10 milliunits/liter of TSH plus 10 μM MS437 or vehicle (DMSO, 0.1%). The cells were then re-fed under the same conditions in serum-free bathing media, which were finally collected either at 2 (MS437) or 3 days (vehicle). For experiments studying the effects of dibutyryl-cAMP, PCCCL3 cells were seeded as above. After 24 h, the cells were grown in complete medium with 10 milliunits/liter of TSH ± 0.1 mM dibutylryl cAMP. The media were changed on days 2 and 4, and the cells then re-fed under the same conditions in serum-free media, which were finally collected either at 2 (dibutyryl cAMP) or 3 days (negative control). For experiments studying the mouse mAb KSAb1 TSHR-stimulating immunoglobulin (39), the PCCCL3 cells were seeded at 25,000 cells/well in 48-well plates. After 24 h, the cells were grown for 5 days in complete medium containing 10 milliunits/liter of TSH ± purified KSAb1. The cells were then re-fed under the same conditions in serum-free media, which were finally collected either at 1 day (KSAb1-stimulated) plus further days in fresh medium (this is referred to as “KSAb1 washout” for cells that had previously been KSAb1-stimulated) or 3 days (negative control).

Human sera were collected from patients with Graves’ disease, or controls, with their consent under an approved Institutional Review Board (IRB) research protocol. For treatment of PCCCL3 cells with human sera, the cells were initially plated as in the KSAb1 experiments described above. After 24 h, the cells were grown in complete medium containing 10 milliunits/liter of TSH at either 10 or 100 milliunits/liter of TSH for 5 days and then plated at 100,000 cells/well in 12-well plates. For mRNA measurements, cells were then either continued in basal media or shifted to complete media containing either 100 or 1000

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milliunits/liter of TSH for 48 h. RNA was purified, reverse-transcribed, and qPCR was performed in duplicate using a StepOnePlus thermal cycler (Applied Biosystems) using SYBR Green and the following primers: Fam20C, 5′-gaggcacaatg-3′ and 5′-gaggctctggagag-3′; and HPRT1, 5′-ctc-attgactttggtg-3′ and 5′-gagggtcaagagttta-ac-3′. Data were analyzed by comparative Cₚ (ΔΔCₚ). For knockdown of Fam20C, PCCL3 cells grown in complete media containing 1,000 milliunits/liter of TSH were plated at 100,000 cells/well in 12-well plates. After 24 h 10 nm Fam20C siRNA (ID304334, Origene) or scrambled duplex oligonucleotide were transfected using RNAiMAX according to the manufacturer’s instructions. After 6 h, media were replaced with complete media containing 1,000 milliunits/liter of TSH for 18 h. At that time, the cells were re-fed under the same conditions in serum-free bathing media, which were finally collected after 48 h. The bathing media were used for *in vitro* iodination of TG and the lysed cells were used for RNA purification followed by qPCR.

**Primary human thyrocyte culture and treatments**

Normal human thyroid tissue was obtained from a patient undergoing total thyroidectomy for thyroid cancer at the National Institutes of Health Clinical Center. The patient provided informed consent on an approved IRB research protocol and materials were received anonymously with approval of research activity through the Office of Human Subjects Research, National Institutes of Health. Primary thyrocytes were prepared and propagated as described (54).

Human thyrocytes were plated in DMEM, 10% FBS plus penicillin/streptomycin at 100,000 cells/well in 12-well plates. After 24 h, the cells were cultured for 1 day in serum-free medium plus 0.1% BSA, and after one further day, the cells were re-fed and cultured for 6 days with DMEM plus penicillin/streptomycin and TSH at a concentration of 100 or 1,000 milliunits/ml. These conditions were tested in two biological replicates, and results in the replicates were identical.

**In vitro enzymatic iodination of TG**

Iodination *in vitro* (55) included lactoperoxidase (30 ng/μl), glucose (2 μg/μl), glucose oxidase (0.352 ng/μl), 100 μM NaI, and 50–250 ng/μl of thyroidal protein. Incubations were initiated with the addition of the glucose oxidase, incubated for 2 h at 37 °C, and stopped by addition of gel sample buffer and boiling for 5 min. To roughly normalize the amount of TG protein in the samples being compared in each experiment, when required we diluted thyrocyte-secreted protein with a known quantity of protein from serum-free medium bathing 293T cells that do not express TG. However, in pilot experiments we found that normalizing the TG protein content may not be necessary as it did not affect the ratio of T₃ formed per unit TG.

**Western blotting**

Samples (2–4 μg of total protein per lane) were subjected to SDS-PAGE under either reducing (Figs. 1–3) or nonreducing conditions (all other figures). Pre-stained molecular mass markers as well as WT TG (330 kDa) were run in lanes adjacent to the experimental samples. Electrotransfer to nitrocellulose was performed for 7 min at 20 V using the iBlot transfer apparatus (Invitrogen). Blocking was performed for 30 min at room temperature with 5% BSA in TBS plus 0.05% Tween 20 (TBS-T) and washed with TBS-T. Primary mouse mAB anti-T₃ was diluted at 1:1000 containing (unless otherwise indicated) 500 ng/ml of free T₃ (to eliminate any possibility of T₄ cross-reactivity) and incubated overnight at 4 °C. Primary rabbit polyclonal anti-TG was diluted 1:5000 in 5% BSA/TBS-T and incubated for 1 h at room temperature. Species-specific HRP-conjugated secondary antibodies (1:5000 dilutions in blocking buffer) were incubated for 30 min at room temperature. Bands were visualized using the WesternBright Sirius kit as directed by the manufacturer (Advansta). Images were captured in a Fotodyne work station with a digital camera; exposure times averaged 20 s for anti-T₃ blots and 5 s for anti-TG blots.

**Quantitation of T₃/TG band intensity ratio**

Band intensities were quantified using ImageQuant 5.2 (Molecular Dynamics). The ratio of intensities of the bands corresponding to the T₃ immunoreactivity within the TG band to the direct TG immunoreactivity from polyclonal anti-TG within the same band was calculated, with the control value set to 1.0. In Fig. 5D, the mean of control values was set to 1.0.

**Data analysis**

Statistical analyses were done using unpaired Student’s *t* test with two-tailed *p* value (Figs. 3–5B and 6) or by one-way ANOVA followed by Dunnett’s test (Figs. 5, A and D, and 7). The level of significance for all statistical tests was set to *p* < 0.05. Statistical values were calculated with GraphPad Prism version 6. Data are presented as mean ± S.D.

**Author contributions**

C. E. C., B. V., S. J. M., V. A. G., S. A., and Y. M. performed experiments, R. L., M. C. G., T. J. S., and P. A. designed experiments, and C. E. C. and P. A. interpreted data. All authors reviewed the results and approved the final submitted version of the manuscript.

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**Note added in proof**

In the version of this article that was published as a Paper in Press on July 25, 2017, some incorrect panels were inadvertently used to assemble Fig. 1A. This error has now been corrected and does not affect the results or conclusions of this work.

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