



Relationship between the dimerization of thyroglobulin and its ability to form triiodothyronine

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Thyroglobulin (TG) is the most abundant thyroid gland protein, a dimeric iodoglycoprotein (660 kDa). TG serves as the protein precursor in the synthesis of thyroid hormones tetraiodothyronine (T₄) and triiodothyronine (T₃). The primary site for T₃ synthesis in TG involves an iodotyrosine acceptor at the antepenultimate Tyr residue (at the extreme carboxyl terminus of the protein). The carboxyl-terminal region of TG comprises a cholinesterase-like (ChEL) domain followed by a short unique tail sequence. Despite many studies, the monoiodotyrosine donor residue needed for the coupling reaction to create T₃ at this evolutionarily conserved site remains unidentified. In this report, we have utilized a novel, convenient immunoblotting assay to detect T₃ formation after protein iodination *in vitro*, enabling the study of T₃ formation in recombinant TG secreted from thyrocytes or heterologous cells. With this assay, we confirm the antepenultimate residue of TG as a major T₃-forming site, but also demonstrate that the side chain of this residue intimately interacts with the same residue in the apposed monomer of the TG dimer. T₃ formation in TG, or the isolated carboxyl-terminal region, is inhibited by mutation of this antepenultimate residue, but we describe the first substitution mutation that actually increases T₃ hormonogenesis by engineering a novel cysteine, 10 residues upstream of the antepenultimate residue, allowing for covalent association of the unique tail sequences, and that helps to bring residues Tyr²⁷⁴⁴ from apposed monomers into closer proximity.

In normal humans, the thyroid gland provides thyroid hormone synthesis and secretion sufficient for 100% of the body's daily supply of thyroxine (T₄)² and ~21% of daily tri-iodothyronine (T₃, the remaining portion coming from deiodination of T₄ (1)). However, a much higher T₃ contribution is thyroid

protein-derived in normal rats (2) and more importantly, in human patients with Graves' disease (3–5). The thyroglobulin protein (TG: >2745 residues) is the primary if not exclusive original source of thyroid hormone production in all vertebrates (6).

Intriguingly, despite ~70 Tyr residues spread along the length of the TG polypeptide, the majority of T₄ has been reported to be formed at the very first tyrosine just five residues from the amino terminus (residue number 1 begins after signal peptide cleavage), whereas the majority of T₃ has been reported to be formed at the very last tyrosine just three residues from the carboxyl terminus (7). Both T₄ and T₃ formation involve a coupling reaction between an acceptor di-iodotyrosine (DIT), which becomes the site of hormone formation within TG (8), and a donor iodotyrosine (either DIT or mono-iodotyrosine (MIT) for T₄ and T₃ formation, respectively) that is converted into dehydroalanine or pyruvate (9). There is good evidence suggesting that much of the T₄ formation involves intra-monomer coupling between the Tyr⁵ acceptor and a Tyr¹³⁰ donor (10, 11); however, the MIT donor responsible for T₃ formation at the antepenultimate residue (*i.e.* the third-to-last residue of TG falling within the sequence SYSK) has remained unclear. Based on mass spectrometry analysis of a lightly iodinated carboxyl-terminal fragment of bovine Tg, Cetrangolo *et al.* (12) reported that Tyr²⁵²² (equivalent to Tyr²⁵¹⁹ of mouse TG) and Tyr²⁵⁵⁵ (equivalent to Tyr²⁵⁵² of mouse TG) form exclusively MIT, and these authors have suggested that both residues could be potential donors in T₃ formation at Tyr²⁷⁴⁸ (Dedieu *et al.* (13) used mass spectrometry of mouse TG to confirm the donor site at Tyr²⁵¹⁹ but argued in contrast that Tyr²⁵⁵² was a probable T₃ acceptor site). Interestingly, however, neither of these mass spectrometry studies considered the possible significance that the conserved antepenultimate Tyr residue itself abundantly forms both MIT (making it a possible donor residue in T₃ formation) and DIT (as expected for an acceptor residue) (12).

The *Tgn* gene encodes a single polypeptide synthesized in the thyrocyte endoplasmic reticulum as a ~330 kDa glycoprotein comprised of repeat domains within region I (bearing type 1 repeats plus linker and hinge segments), region II + III (bearing type 2 and type 3 repeats), the carboxyl-terminal cholinesterase-like (ChEL) domain (containing both of the potential donor MIT residues noted above), and a unique (~50 residue) tail sequence bearing the antepenultimate Tyr residue that can

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² The abbreviations used are: T₄, thyroxine; TG, thyroglobulin; ChEL, cholinesterase-like; T₃, tri-iodothyronine; TSH, thyroid-stimulating hormone; DIT, di-iodotyrosine; MIT, mono-iodotyrosine; DMEM, Dulbecco's modified Eagle's medium; HRP, horseradish peroxidase; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; mTG, mouse TG.

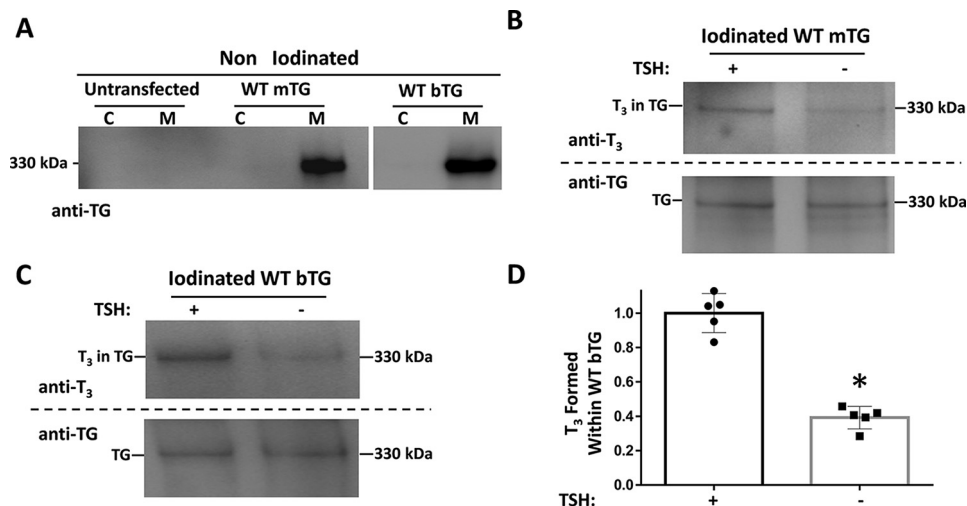


Figure 1. The stimulatory effect of TSH on intrinsic ability for *de novo* T_3 formation within endogenous secreted TG is also observed for recombinant secreted TG. A, PCCL3-clone 7F bearing homozygous disruption of the *Tgn* gene was either untransfected or stably transfected to express WT mouse TG (*mTG*) or WT bovine TG (*bTG*) (see “Experimental procedures”). After 1 day of culture in serum-free media, the cells were washed and re-fed for 4 contiguous days with serum-free media (*M*) that were collected, and the cells (*C*) were lysed. Media and cell lysates were analyzed by SDS-PAGE, electrotransfer to nitrocellulose, and immunoblotting with anti-TG Ab. (TG from WT mouse thyroid was run in an adjacent lane as a 330-kDa molecular mass marker for panels A–C.) B, PCCL3-clone 7F cells stably expressing *mTG* were cultured as in panel A in the presence or absence of TSH. Samples were iodinated *in vitro* as previously described (5) followed by SDS-PAGE (4 μ g of protein per lane), electrotransfer to nitrocellulose, and immunoblotting with mAb anti- T_3 and anti-TG as indicated. C, PCCL3-clone 7F cells stably expressing *bTG* were cultured as in panel A in the presence or absence of TSH. Samples were analyzed as in panel B. D, quantitation of relative T_3 formation in bTG from five independent samples; mean \pm S.D.; *, $p < 0.05$ comparing the two conditions.

form T_3 and is conserved throughout all vertebrates from lamprey to human (14). Shortly after its synthesis, TG self-associates noncovalently into a \sim 660 kDa TG dimer (15).

Although the interface between the monomeric TG subunits is incompletely understood, there must be regions of intimate contact, because upon oxidation in the lumen of thyroid follicles, inter-monomeric covalent bonds are created that include the formation of disulfide pairs (16–19) as well as dityrosine bridges (20, 21). Although the inter-monomer contact surface may not be limited to ChEL, this domain has been found to be both necessary and sufficient for TG dimerization, as it contributes two α helices to a 4-helix bundle needed for homodimerization of acetylcholinesterase and its evolutionary homologs (22). Homodimerization brings the contiguous unique tail sequences (bearing the antepenultimate Tyr residues from closely-apposed TG monomers) into proximity (22).

Classic studies have shown that thyroid peroxidase shows no marked specificity in its ability to catalyze TG iodination or thyroid hormone formation over that of lactoperoxidase or myeloperoxidase (23) (whereas efficient T_4 and T_3 formation requires the TG substrate in its native conformation (8)); therefore, multiple laboratories have pursued *de novo* T_3 formation upon *in vitro* iodination of TG isolated from thyroid glands of various animal species. We recently re-designed this assay to develop a simple and specific means to detect *de novo* T_3 formation after *in vitro* iodination of TG secreted from thyrocyte cell lines grown in culture (5). In the current study, we have further adapted the method to permit analysis of T_3 formation in recombinant TG molecules that bear specifically-engineered mutations within critical hormonogenic Tyr residues, or other residues in the TG molecule. This approach has allowed us to explore the relationship between TG dimerization and its efficiency in T_3 formation at the antepenultimate Tyr residue that has been conserved in evolution for 500 million years.

Results

Recombinant TG functions like endogenous TG in T_3 formation

Thyrocyte cell lines growing as monolayer cultures cannot iodinate and form thyroid hormone within their own secreted TG; however, upon *in vitro* iodination of TG secreted into cell culture medium, *de novo* T_3 formation can be measured by immunoblotting with a mAb that recognizes T_3 , in parallel with immunoblotting using a polyclonal anti-TG antibody (5). We wished to establish that recombinant TG expressed after transfection of cultured cells remains a physiologically-relevant substrate for T_3 hormonogenesis. With this in mind, using CRISPR-Cas9, we first engineered a PCCL3 (rat thyrocyte) cell line with knock-out of endogenous TG expression; these cells expressed no detectable TG protein either in cells (*C*) or medium (*M*, Fig. 1A). In contrast, transfection of these cells with either recombinant mouse TG or bovine TG led to TG secretion into the medium (Fig. 1A) and upon iodination, both recombinant mouse TG (Fig. 1B) and bovine TG (Fig. 1C) readily formed T_3 . T_3 formation in TG did not require TSH stimulation of thyrocytes, but we confirmed that T_3 formation upon iodination was even greater when the recombinant TG was secreted from thyrocytes that had been stimulated by TSH (Fig. 1, B–D), consistent with what has been observed for endogenously expressed TG (5). Thus we conclude that recombinant TG behaves as a physiological substrate for studies of hormonogenesis of T_3 .

The role of the antepenultimate Tyr²⁷⁴⁴ in *de novo* T_3 formation within mouse TG

We and others have previously shown that the carboxyl-terminal fragment of TG is particularly enriched in T_3 (5). As noted above, three Tyr residues of mouse TG: Tyr²⁵¹⁹, Tyr²⁵⁵², and Tyr²⁷⁴⁴, have all been implicated in T_3 hormonogenesis.

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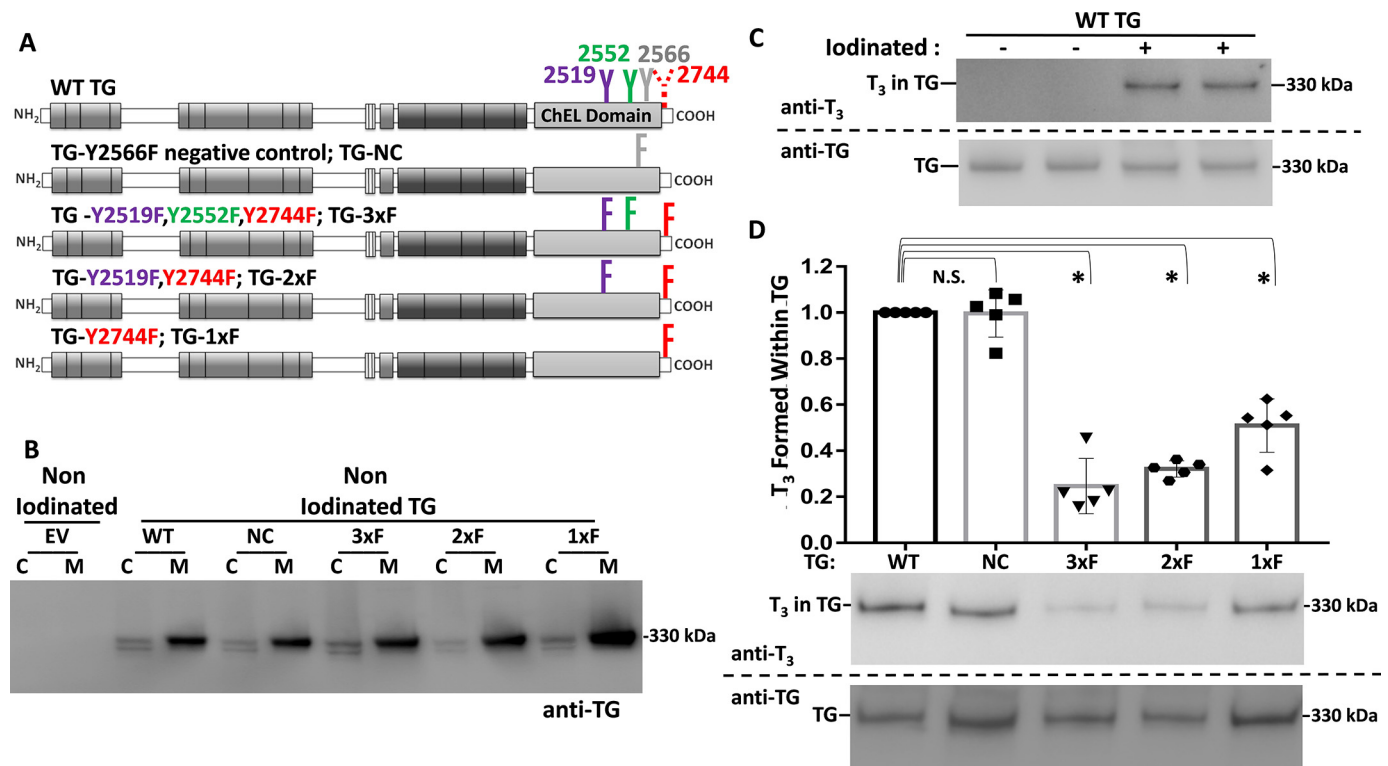


Figure 2. De novo T₃ formation in TG mutagenized to abolish selected iodination target sites. *A*, WT mTG (WTTG) bears ~70 Tyr residues including Tyr²⁵¹⁹, Tyr²⁵⁵², Tyr²⁷⁴⁴, and Tyr²⁵⁶⁶ (the latter residue has not been implicated in thyroid hormone formation). We engineered TG-Y2744F (TG-1xF) as a single missense mutant plus Y2519F (TG-2xF) as a double missense mutant plus Y2552F (TG-3xF) as a triple missense mutant, with TG-Y2566F as a negative control (NC). The mutagenic primers used to create these mutants are shown in Table 1. *B*, 293T cells were transfected either with empty vector (EV) or with the TG constructs indicated in panel *A*. Serum-free media were collected for 24 h (M) and the cells (C) were lysed as described under "Experimental procedures." The samples were analyzed by SDS-PAGE (2 μg of protein per lane), electrotransfer to nitrocellulose, and immunoblotting with anti-TG. *C*, recombinant WT TG secreted from transfected 293T cells into serum-free bathing media was either noniodinated or iodinated *in vitro*. Samples for SDS-PAGE (2 μg of protein per lane) were analyzed as described in the legend to Fig. 1, *B* and *C*, indicating successful T₃ formation in recombinant TG secreted from the heterologous cells. *D*, below, the indicated TG constructs, secreted from 293T cells, were iodinated *in vitro* and analyzed by SDS-PAGE and immunoblotting as in panel *C*; above, quantitation of relative T₃ content of each construct after iodination, from 5 independent experiments; mean ± S.D.; *, *p* < 0.05 compared with WT TG; NS, not significant.

Therefore, using site-directed mutagenesis, we sequentially replaced hormonogenic Y2744F (TG-1xF), plus Tyr²⁵¹⁹ (TG-2xF), plus Tyr²⁵⁵² (TG-3xF). A fourth Tyr residue at position 2566 has not been similarly implicated, thus Y2566F is considered to be a negative control in studies of T₃ hormonogenesis (Fig. 2A). When expressed in 293T cells, each of these recombinant TG proteins was efficiently delivered from cells (C) to medium (M, Fig. 2B). Importantly, recombinant WT mouse TG secreted from 293T cells readily formed T₃ upon *in vitro* iodination (Fig. 2C). In contrast, the single TG-1xF mutant exhibited a significantly diminished ability to form T₃ upon iodination, and TG-2xF and TG-3xF mutants showed small further defects in T₃ formation (Fig. 2D). In 5 additional independent samples comparing three single TG-1xF mutants (Y2519F, Y2552F, or Y2744F), we observed that TG-Y2519F exhibited no inhibition of T₃ formation, TG-Y2552F exhibited a tendency to ~30% decreased T₃ formation (but did this did not achieve statistical significance), whereas TG-Y2744F showed a significant 50% decrease in T₃ formation (data not shown). Altogether, these data suggest that TG-Y2744 is an important contributor to T₃ hormonogenesis that takes place within the carboxyl-terminal region of TG.

Recent studies of the lamprey *Tgn* gene sequence indicate that the ChEL domain and the contiguous unique tail sequence of TG that together bear the three Tyr (noted above)

that have been implicated in T₃ hormonogenesis are evolutionarily conserved throughout the vertebrates (14). We have previously reported that, when led by a conventional signal sequence, the ChEL domain with contiguous unique tail sequence (so-called "secretory ChEL") is rapidly folded in the endoplasmic reticulum and efficiently secreted from cells to medium in the absence of the main portion of the TG protein comprised of regions I–II–III (24). To determine whether the ChEL domain with contiguous unique tail sequence is competent for T₃ hormonogenesis in the absence of region I–II–III, we expressed FLAG-tagged versions of secretory ChEL in 293T cells and collected the secretion in preparation for iodination *in vitro*. Of these constructs, Y2744F (ChEL-1xF), plus Y2519F (ChEL-2xF), plus Y2552F (ChEL-3xF, Fig. 3A) were each well-secreted from cells to medium, as was the Y2566F negative control, when detected by immunoblotting with either anti-FLAG or anti-Tg (Fig. 3B). Remarkably, iodination of WT secretory ChEL led to T₃ formation (Fig. 3C). Once again, the single ChEL-1xF mutant exhibited a markedly diminished ability to form T₃ upon iodination, and ChEL-2xF and ChEL-3xF mutants showed only small incremental defects in T₃ formation (Fig. 3D). These data strongly suggest that T₃ hormonogenesis involves one or more donor MIT residues residing within the ChEL domain or its contiguous unique tail sequence, coupling to the DIT acceptor at Tyr²⁷⁴⁴.

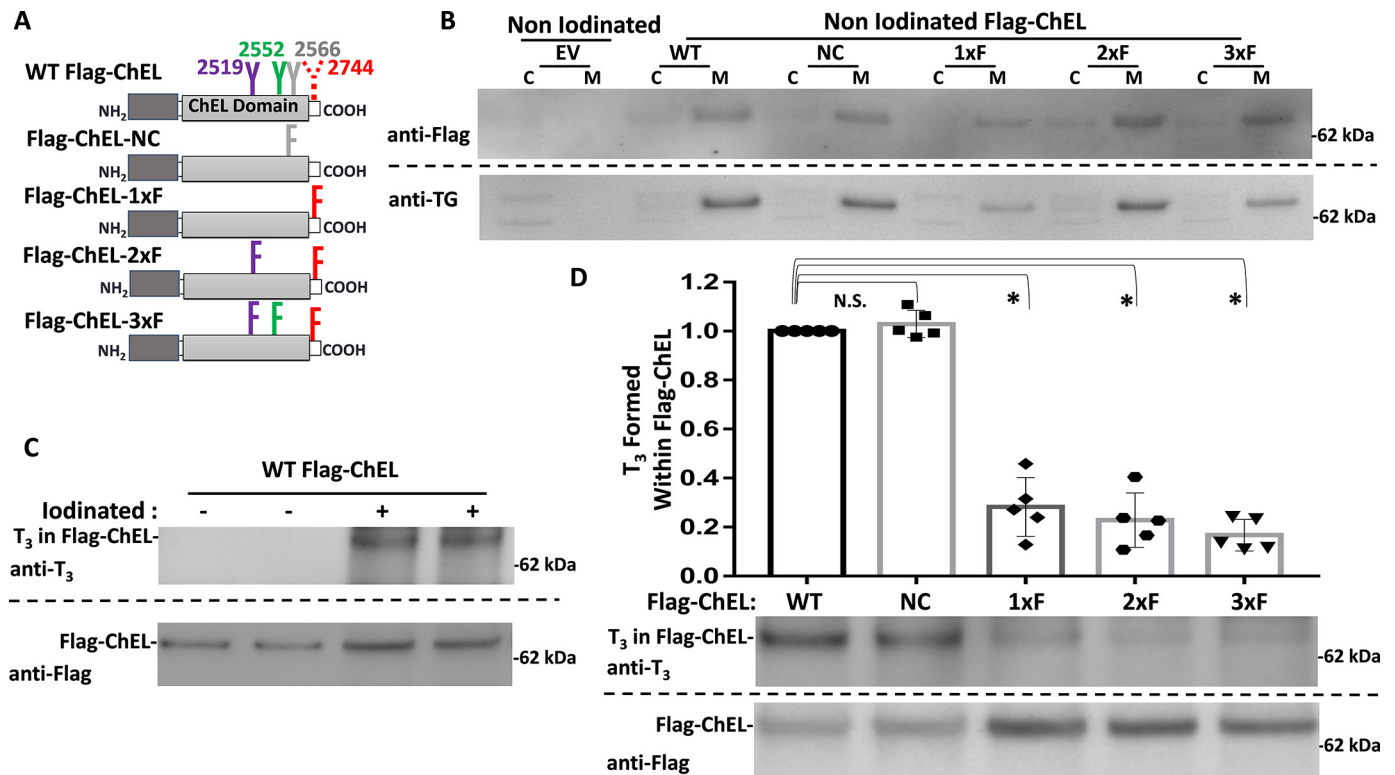


Figure 3. *De novo* T_3 formation in secretory FLAG-ChEL and its contiguous unique tail sequence, mutagenized to abolish selected iodination target sites. *A*, secretory ChEL and its contiguous unique tail sequence bears the same Tyr residues as those described in the legend to Fig. 2. Dark gray boxes, a FLAG peptide was engineered after the signal peptide of secretory ChEL. Using this, we engineered FLAG-ChEL-Y2744F (*Flag-ChEL-1xF*) as a single missense mutant plus Y2519F (*Flag-ChEL-2xF*) as a double missense mutant plus Y2552F (*Flag-ChEL-3xF*) as a triple missense mutant, with FLAG-ChEL-Y2566F as a negative control (NC). *B*, 293T cells were transfected either with empty vector (EV) or with the indicated FLAG-ChEL constructs. The samples were processed as described in the legend to Fig. 2B, and immunoblotted with anti-FLAG and anti-TG Abs as indicated. *C*, recombinant WT FLAG-ChEL secreted from transfected 293T cells into serum-free bathing media was first immunoprecipitated with anti-TG and then either noniodinated or iodinated *in vitro*, as indicated. Samples for SDS-PAGE were analyzed by immunoblotting with mAb anti- T_3 and anti-FLAG Abs as indicated, demonstrating successful T_3 formation in the recombinant secretory ChEL with contiguous unique tail sequence. The position of a 62-kDa molecular mass marker is indicated. *D*, below: the indicated FLAG-ChEL constructs, secreted from 293T cells, were processed as in panel C. Above, quantitation of relative T_3 content of each construct after iodination, from 5 independent experiments; mean \pm S.D.; *, $p < 0.05$ compared with WT FLAG-ChEL; NS, not significant.

Interaction between the side chains of residue 2744 and 2744 of adjacent TG monomers

Because TG undergoes tail-to-tail dimerization (22), we considered the possibility that T_3 formation could involve side chain interactions between a donor MIT-2744 and a DIT-2744 at the conserved antepenultimate T_3 acceptor site. If so, we reasoned that a mutant TG-Y2744C might use the novel Cys residue to form an inter-monomer disulfide bond. With this in mind, we expressed three substitution mutants: TG-Y2519C, TG-Y2522C, or TG-Y2744C (Fig. 4A). Of these, only TG-Y2744C, but not TG-Y2519C or TG-Y2522C, formed a covalent homodimer as analyzed by Western blotting after nonreducing SDS-PAGE (Fig. 4B). We also constructed TG-2xC (double) and TG-3xC (triple) mutants (Fig. 4A); all such mutants could form a covalent homodimer as long as they included the TG-Y2744C residue. In contrast, a TG-Y2519C, Y2552C double mutant could not form a covalent homodimer (data not shown).

The primary structure of Tg ordinarily includes 122 Cys residues, 6 of which are in the ChEL domain to be engaged in three evolutionarily conserved intra-domain disulfide bonds (25, 26). Although unlikely, we considered the possibility that disulfide cross-linking of the TG-Y2744C mutant could involve a side chain interaction between the novel engineered Cys residue

and that of a preexisting upstream TG Cys residue. To confirm the presence of a direct Cys²⁷⁴⁴-Cys²⁷⁴⁴ disulfide, we introduced the Y2744C mutation into the FLAG-tagged secretory ChEL with contiguous unique tail sequence (Fig. 5A). Upon expression in 293T cells, it was immediately apparent that the Y2744C side chain conferred upon the ChEL partners the ability to make a covalent dimer (Fig. 5B). To demonstrate that it is Y2744C and not some other TG residue required for the covalent interaction, we co-transfected FLAG-tagged secretory ChEL-Y2744C with TG-Y2744C in 293T cells. Secreted proteins were subjected to immunoprecipitation with anti-FLAG antibody that exclusively recognizes the ChEL partner, boiled in 1% SDS to denature all proteins and dissociate all noncovalently associated proteins, and then diluted into SDS-free buffer to reconstitute our usual immunoprecipitation buffer. Repeat immunoprecipitations with anti-FLAG were analyzed by nonreducing and reducing SDS-PAGE and Western blotting with anti-TG antibody. When expressed alone or with WT-TG lacking the Y2744C mutation, FLAG-ChEL-Y2744C was recovered both as a monomer and as a covalent homodimer (Fig. 5C). However, when co-expressed with TG-Y2744C, FLAG-ChEL-Y2744C was recovered not only as a monomer and covalent homodimer, but also as a covalent heterodimer with TG-Y2744C (Fig. 5C). Both covalent

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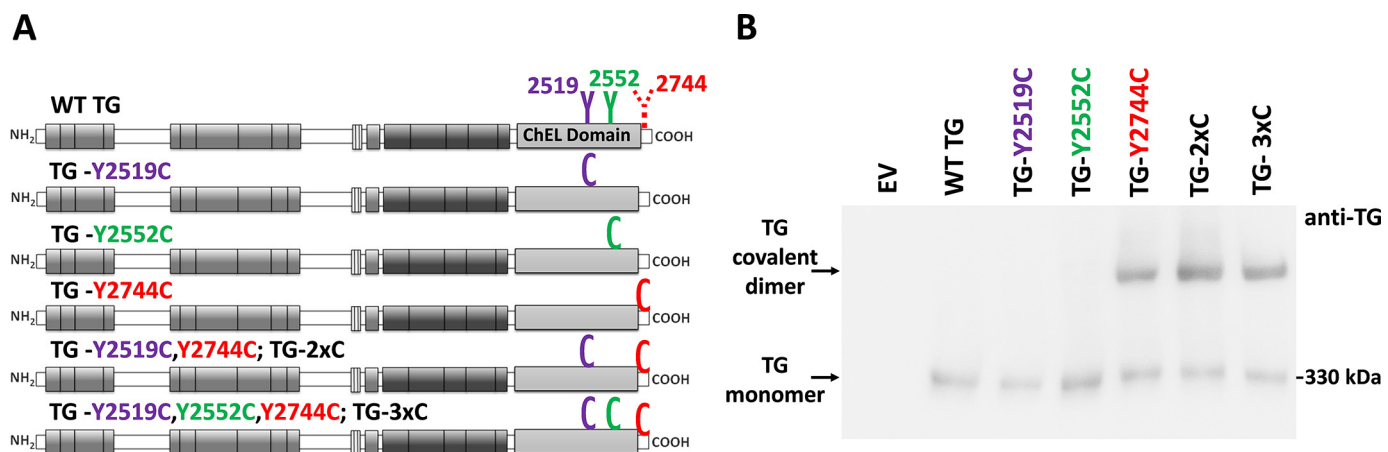


Figure 4. Interaction at the antepenultimate residue between monomeric subunits of the TG homodimer. *A*, we engineered TG-Y2519C, TG-Y2552C, TG-Y2744C, or TG-Y2519C plus Y2744C (TG-2xC) as a double mutant, plus Y2552C (TG-3xC) as a triple mutant. *B*, 293T cells were transfected either with empty vector (EV) or with the indicated FLAG-ChEL constructs. Serum-free bathing media were collected for 24 h and analyzed by nonreducing SDS-PAGE, electrotransfer to nitrocellulose, and immunoblotting with anti-TG. All constructs bearing the Y2744C substitution formed an intermolecular disulfide bond between TG subunits, as indicated.

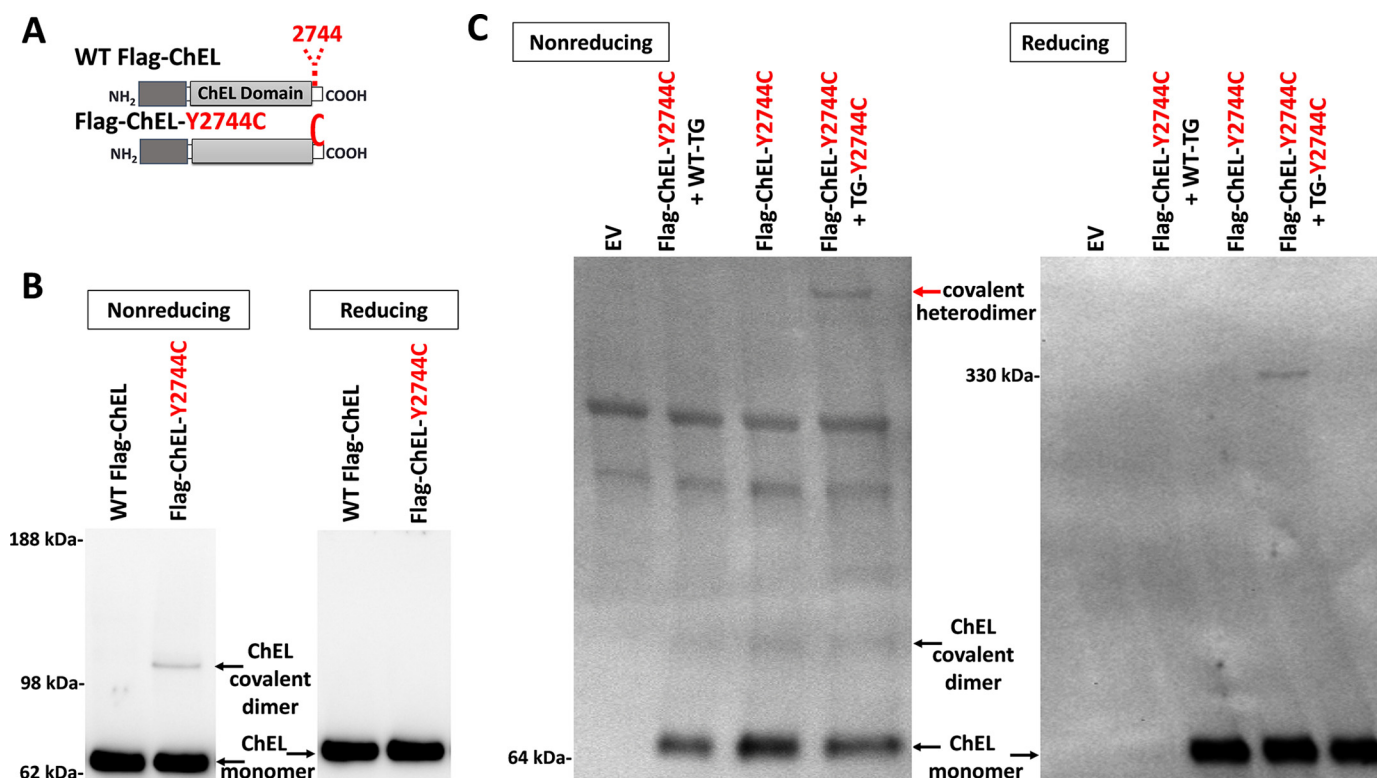


Figure 5. Interaction of residue 2744 of the unique tail sequence in apposed monomeric subunits of TG. *A*, dark gray boxes signify signal peptide plus FLAG peptide of secretory ChEL; we engineered FLAG-ChEL-Y2744C as a single substitution. *B*, 293T cells were transfected either with the WT or the indicated mutant FLAG-ChEL constructs, bathing media (M) was collected for 24 h, and the samples were analyzed by SDS-PAGE under nonreducing or reducing conditions, electrotransfer to nitrocellulose, and immunoblotting with mAb anti-FLAG. The ChEL construct bearing the Y2744C substitution in the unique tail sequence formed an intermolecular disulfide bond, as indicated. *C*, 293T cells were co-transfected (1:1 molar ratio) either with empty vector or with the two constructs indicated. The bathing media were immunoprecipitated with anti-FLAG M2, denatured in boiling SDS, diluted with SDS-free immunoprecipitation buffer, and re-immunoprecipitated with fresh anti-FLAG before analysis by nonreducing (*left panel*) or reducing (*right panel*) SDS-PAGE and immunoblotting with anti-TG. WT TG was run in an adjacent lane as a 330-kDa molecular mass marker (not shown). Note that FLAG-ChEL-Y2744C formed a covalent heterodimer only when co-expressed with TG-Y2744C but not with WT TG.

homodimers and heterodimers were dissociated under reducing conditions, releasing TG-Y2744C from FLAG-ChEL-Y2744C (Fig. 5C). The data indicate that upon dimerization, the side chain of TG residue 2744 can find the apposed TG 2744 for intimate interaction. Nevertheless, by mutating the antepenultimate residue, the Y2744C mutation

would be expected to inhibit T_3 formation despite promoting a covalent homodimeric partnership. Indeed, upon iodination, T_3 formation was significantly inhibited in both FLAG-ChEL-Y2744C (Fig. 6A) and TG-Y2744C (Fig. 6B). The fact that residual T_3 is still formed within the TG-Y2744C mutant is consistent with published reports that

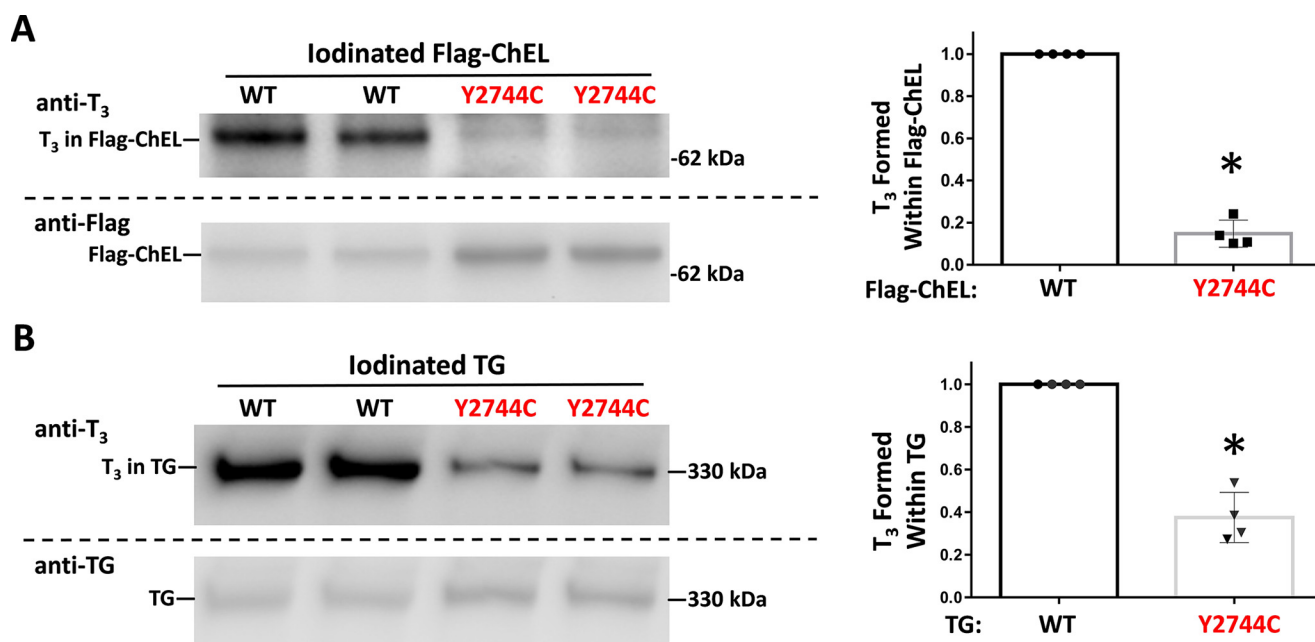


Figure 6. Y2744C substitution strongly inhibits *de novo* T_3 formation either in FLAG-ChEL or TG. A, FLAG-ChEL or FLAG-ChEL-Y2744C secreted from 293T cells was iodinated *in vitro* and analyzed for T_3 formation as described in the legend to Fig. 3D. The position of a 62-kDa molecular mass marker is indicated. Quantitation of relative T_3 content of each construct after iodination, from four independent samples; mean \pm S.D.; *, $p < 0.05$ when comparing the two kinds of ChEL samples is shown. B, WT TG or TG-Y2744C secreted from 293T cells was iodinated *in vitro* and analyzed for T_3 formation as described in the legend to Fig. 2D. Quantitation of the relative T_3 content of each construct after iodination, from four independent experiments; mean \pm S.D.; *, $p < 0.05$, when comparing the two kinds of TG samples is shown.

Tyr²⁷⁴⁴ is not the exclusive site of T_3 formation, as secondary sites are also utilized (7).

The foregoing data are all consistent with the notion that T_3 hormonogenesis at the conserved antepenultimate Tyr residue of TG involves contact between the 2744 side chains of closely apposed TG monomers. However, we noted that (similar to authentic acetylcholinesterase) the percent of secretory ChEL or TG molecules with a covalent Cys²⁷⁴⁴-Cys²⁷⁴⁴ disulfide bond was only a fraction of total, suggesting that the 2744 side chain within the unique tail sequence of each TG monomer may have only limited contact time or proximity with the apposed 2744 side chain. With this in mind, we reasoned that introduction of a nearby Cys residue to allow for nearby covalent “stapling” of the tail sequences in the dimer might increase contact between apposed Tyr²⁷⁴⁴ side chains. We therefore introduced a single S2734C substitution mutation into FLAG-tagged secretory ChEL, just 10 residues away from the conserved T_3 hormonogenic site (Fig. 7A). As expected, the added tail Cys residue resulted in covalent dimerization of secretory ChEL (Fig. 7B). Remarkably, upon *in vitro* iodination, secretory ChEL-S2734C yielded significantly more T_3 formation than that produced by WT ChEL (Fig. 7C). Taken together, the data strongly suggest that the conserved antepenultimate T_3 hormonogenic site undergoes a MIT-DIT coupling reaction through the interactions of apposed monomers within the TG dimer.

Discussion

In a classic study of thyroid hormonogenesis at the evolutionarily conserved T_4 -forming site in TG, it was shown that, upon iodination, an individual polypeptide fragment comprised solely of the first 171 residues of TG could form T_4 at

Tyr⁵, whereas Tyr¹³⁰ had lost its phenolic side chain (11). As there is no evidence that such a polypeptide fragment is dimeric (27), these data imply that the evolutionarily conserved T_4 -forming site in TG involves a DIT donor at position 130 coupled to a DIT acceptor at position 5 of the same TG monomer; this conclusion has been supported by other work (10) including by mutagenesis of specific Tyr residues within the amino-terminal polypeptide of TG (28).

Understanding the structural basis for T_3 formation at the antepenultimate TG residue is of considerable physiological importance because (a) this is the main site of T_3 hormonogenesis within TG (7); (b) TSH stimulation of thyrocytes physiologically increases the T_3 hormonogenic capability of TG, including at the antepenultimate site (29); (c) this provides a rationale for evolutionary conservation of the antepenultimate Tyr residue in vertebrate TG (14); and (d) enhanced T_3 hormonogenesis within TG is an especially relevant problem in Graves' disease (5, 30). However, there have been no publications to clearly suggest the MIT residue that serves as a primary donor to this evolutionarily conserved T_3 -forming site in TG. In principle, insight into this T_3 -forming donor-acceptor pair should have been obtainable by an unbiased mass spectrometry analysis; however, proteolytic cleavage of TG at Lys²⁷⁴², leaving a carboxyl-terminal SYSK tetrapeptide, was too small to be detected by mass spectrometry; thus, no definitive conclusion could be drawn (13). In this report, using a mutagenesis approach, we present evidence to support the hypothesis that T_3 can be formed at the antepenultimate residue of mouse TG by coupling a DIT acceptor at position 2744 with an MIT donor at position 2744 from its closely apposed monomer within the TG dimer (Fig. 8).

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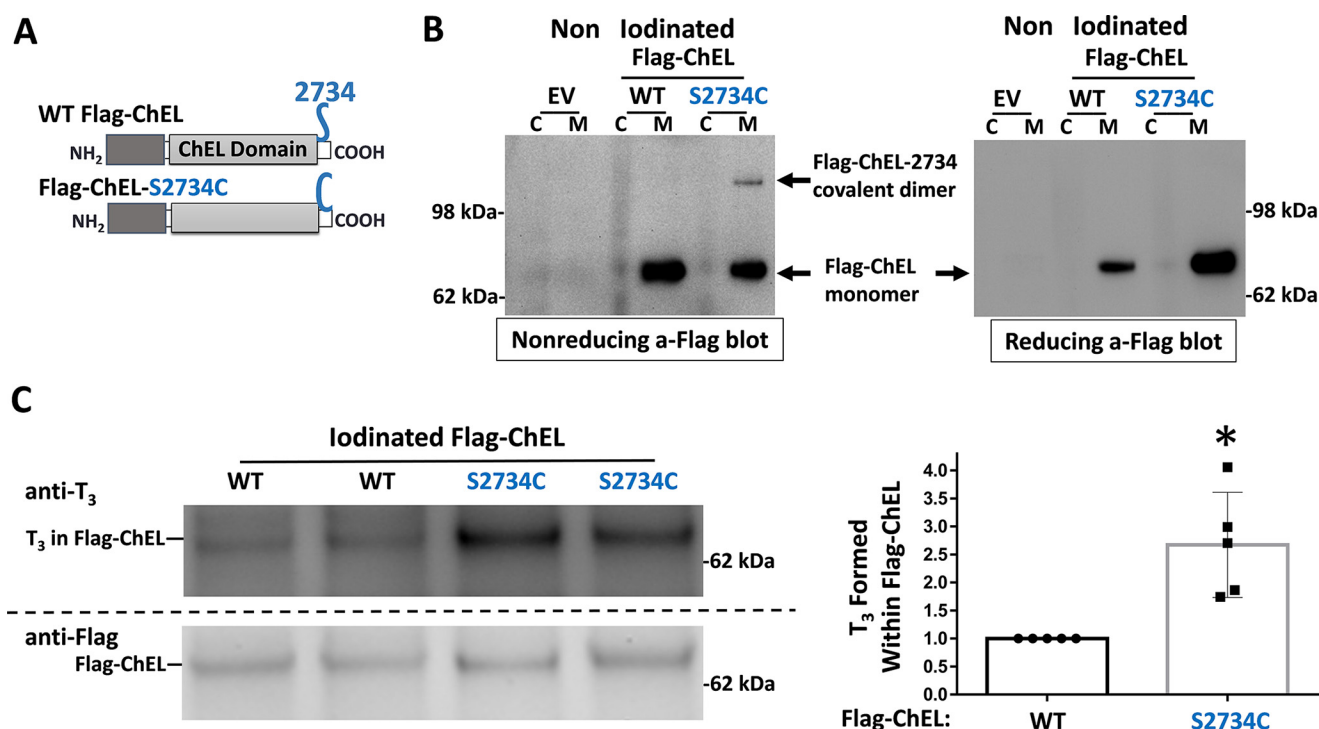


Figure 7. Stimulation of *de novo* T_3 formation upon covalent cross-linking of the apposed unique tail sequences contiguous with FLAG-ChEL. *A*, dark gray boxes signify signal peptide plus FLAG peptide of secretory ChEL; we engineered FLAG-ChEL-S2734C as a single substitution mutant. *B*, 293T cells were transfected either with the WT or the indicated mutant FLAG-ChEL constructs, serum-free bathing media (*M*) were collected for 24 h as described in the legend to Fig. 3B, and the samples analyzed by SDS-PAGE under nonreducing or reducing conditions, electrotransfer to nitrocellulose, and immunoblotting with mAb anti-FLAG. The ChEL construct bearing the S2734C substitution in the unique tail sequence (10 residues upstream from the conserved antepenultimate T_3 site) formed an intermolecular disulfide bond, as indicated. The positions of molecular weight markers are indicated. *C*, FLAG-ChEL or FLAG-ChEL-S2734C secreted from 293T cells was iodinated *in vitro* and analyzed for T_3 formation as described in the legend to Fig. 3D. The position of a 62-kDa molecular mass marker is indicated. Quantitation of relative T_3 content of each construct after iodination, from five independent experiments is shown; mean \pm S.D.; *, $p < 0.05$, when comparing the two kinds of ChEL samples is shown.

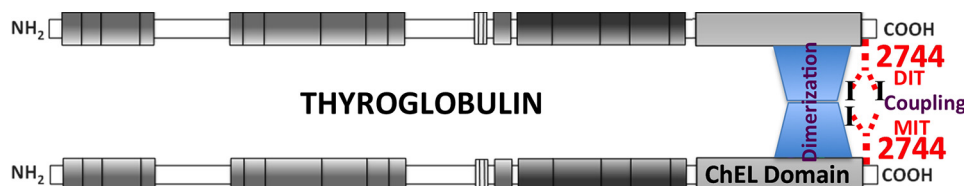


Figure 8. Hypothesis of the mechanism of T_3 formation at the evolutionarily conserved carboxyl-terminal site of TG. Data presented suggest a coupling reaction between a MIT-2744 donor and a DIT-2744 acceptor to form T_3 at the acceptor site in mouse TG.

Using a novel nonradioactive *in vitro* iodination of recombinant mouse TG followed by immunoblotting with mAb anti- T_3 , we confirm that Tyr²⁷⁴⁴ is an important contributor to total T_3 formation within TG (Fig. 2), and, remarkably, it is possible to form T_3 at this site within a foreshortened version of TG bearing only the evolutionarily conserved ChEL domain and its contiguous unique tail sequence (Fig. 3). The ChEL domain could either provide potential MIT donor residues to the DIT acceptor within the unique tail sequence (12) or it could provide the potential for ChEL dimerization (22, 31). Indeed, we demonstrate that mutagenic replacement of Y2744C confers covalent homodimerization of TG (Fig. 4) as well as that of the secretory ChEL with its contiguous unique tail sequence (Fig. 5B). We prove that this is a direct Cys²⁷⁴⁴–Cys²⁷⁴⁴ disulfide bond as demonstrated by the fact that ChEL-Y2744C cannot make a covalent bond with co-expressed TG, but such a covalent bond becomes possible between ChEL-Y2744C and TG-Y2744C (Fig. 5C). Thus, there is intimate contact between

the side chains at residue 2744 of monomer partners within the TG dimer. Of course, introducing the Cys²⁷⁴⁴ missense mutation eliminates the critical Tyr at this position and, not surprisingly, significantly inhibits T_3 formation upon iodination of either ChEL with its contiguous unique tail sequence, or full-length TG (Fig. 6).

Our evidence suggests that TG dimerization brings the neighboring unique tail sequences into closer contact from which a coupling reaction may take place. The longer and more stable the contact between neighboring unique tail sequences, the better should be the likelihood of coupling. In support of this notion, we artificially engineered a “super T_3 -former” by creating a ChEL-S2734C substitution that allows for covalent stapling of the neighboring unique tail sequences just 10 residues from the antepenultimate T_3 site. Although ~120 deleterious mutations in TG have been characterized (32), the S2734C represents the first described mutation (albeit not naturally occurring) that actually enhances thyroid hormonogen-

Table 1
Primers utilized to introduce mutations in mTG and in secretory ChEL domain

Name of the primers	DNA sequence (5'–3') of primers	Position ^a in mTG of the 5' end of primers	Mutation introduced ^b
FP-FLAG-ins	gtggtctccaccggctcgactacaaggacgacgatga caagccttggccaatc	NA ^c	Insertion of N-terminal FLAG peptide DYKDDDDK in secretory ChEL domain
RP-FLAG-ins	gattggaccaaaggctgtcatcgtctcctttagtctc gaccgggtggagaccac	6575	
FP-Y2744F	gcctgtcccgaagagcttcagcaaatgatctagag	8274	Tyr ²⁷⁴⁴ to Phe ²⁷⁴⁴ in mTG and in FLAG-ChEL at equivalent position
RP-Y2744F	ctctagatcattgtgctgaagctcttggggacaggc	NA	
FP-Y2566F	aatgccaccgggactctttatcatctgtctcc	7741	Tyr ²⁵⁶⁶ to Phe ²⁵⁶⁶ in mTG and in FLAG-ChEL at equivalent position
RP-Y2566F	gggacagatgataaagaagctcccgggtggcatt	7773	
FP-Y2552F	gagcactccacagacacttggcctccttc	7696	Tyr ²⁵⁵² to Phe ²⁵⁵² in mTG and in FLAG-ChEL at equivalent position
RP-Y2552F	gaaggaggcaagctgctgtggagtgctc	7725	
FP-Y2519F	ccaacagcaaaaacagccttttccaggcactgc	7595	Tyr ²⁵¹⁹ to Phe ²⁵¹⁹ in mTG and in FLAG-ChEL at equivalent position
RP-Y2519F	gcagtgtcggaaaaggctgttttctgtgtgg	7627	
FP-Y2744C	gcctgtcccgaagagctcagcaaatgatctagag	8274	Tyr ²⁷⁴⁴ to Cys ²⁷⁴⁴ in mTG and in FLAG-ChEL at equivalent position
RP-Y2744C	ctctagatcattgtgctgaagctcttggggacaggc	NA	
FP-Y2552C	gagcactccacagacacttggcctccttc	7696	Tyr ²⁵⁵² to Cys ²⁵⁵² in mTG and in FLAG-ChEL at equivalent position
RP-Y2552C	gaaggaggcagctgctgtggagtgctc	7725	
FP-Y2519C	ccaacagcaaaaacagccttttccaggcactgc	7595	Tyr ²⁵¹⁹ to Cys ²⁵¹⁹ in mTG and in FLAG-ChEL at equivalent position
RP-Y2519C	gcagtgtcggcaaaaggctgttttctgtgtgg	7627	
FP-S2734C	acctggattagaagaagactctcggctcactggag	8238	Ser ²⁷³⁴ to Cys ²⁷³⁴ in mTG and in FLAG-ChEL at equivalent position
RP-S2734C	ctccagtgagcgcagaggtctcttcttaaccaggt	8274	

^a In the DNA numbering, position 1 is A from the start codon of mTG precursor NCBI Reference Sequence: NM_009375.2.

^b Amino acid positions are numbered in the mature protein (*i.e.* after subtracting the 20-amino acid signal peptide of mTG precursor NCBI Reference sequence: NP_033401.2).

^c NA, not applicable.

esis within the T₃-forming region of TG. The extra Cys residue did induce a disulfide bond between the neighboring unique tail sequences (Fig. 7B) along with a marked increase in the ability of the construct to form T₃ upon iodination (Fig. 7C). Once again, these data may provide a rationale for why the unique tail sequence of TG bearing the conserved T₃-forming site has traveled together in evolution with the conserved ChEL domain (14): ChEL provides homodimerization, allowing for MIT–DIT coupling at the antepenultimate Tyr residue of TG.

Experimental procedures

Materials

NaI, lactoperoxidase, glucose oxidase, dextrose, fetal bovine serum, insulin, hydrocortisone, apo-transferrin, TSH, ampicillin, dithiothreitol (DTT), and protein A-agarose were from Sigma. Plasmid purification kits were from Zymo Research. Protease inhibitor mixture was from Roche Applied Science. Penicillin/streptomycin, PBS, DMEM, and DMEM/F-12 were from Gibco. Lipofectamine 2000, hygromycin, lithium dodecyl sulfate gel loading buffer, 4–12% polyacrylamide BisTris SDS-PAGE gels, 6% polyacrylamide Tris glycine SDS-PAGE gels, SeeBlue Plus2 molecular weight markers, LB broth base, and subcloning efficiency DH5 α -competent cells were from Invitrogen; LB agar was from BD Difco, QuikChange Lightning and QuikChange-II mutagenesis kits were from Agilent Technologies; DNA primers were from Integrated DNA Technologies; a linearized hygromycin-resistance gene was from Clontech; Viatec transfection reagent was from Fisher.

Antibodies

Mouse mAb anti-T₃ clone 3A6 ($\leq 0.1\%$ cross-reactivity with T₄ by ELISA) was from Invitrogen, rabbit polyclonal anti-TG was as described (15). Mouse mAb anti-FLAG M2 and anti-FLAG M2 affinity agarose gel were from Sigma; HRP-conjugated goat anti-mouse IgG was from Bio-Rad; HRP-conjugated goat anti-rabbit IgG was from Jackson ImmunoResearch.

Mutagenic primers

Mutagenic primers shown in Table 1 were designed utilizing the QuikChange Primer Design Program. Mouse TG (mTG) cDNA sequences were obtained from NCBI database (mTG NCBI Reference Sequence: NM_009375.2).

Construction of FLAG-tagged ChEL domain

Mutagenic primers FP-FLAG-ins and RP-FLAG-ins (Table 1) were designed to insert a sequence, encoding the FLAG peptide (DYKDDDDK) in the N-terminal of the ChEL domain, right after the prolactin signal peptide in secretory ChEL in the pCMS-EGFP vector (Clontech). Mutagenesis by PCR was performed as described below. Mutants with FLAG insertion were confirmed by DNA sequencing utilizing the FP-N-terminal: 5'-tttgagaagctcagaataaacg-3'.

Mutagenesis by PCR

PCR primers with the indicated point mutations or insertion of the N-terminal FLAG tag were incorporated with the QuikChange PCR mutagenesis kit as per the manufacturer's instructions. Only one set of mutagenic primers was used per reaction; for double and triple mutations, sequential rounds of point mutagenesis were performed. QuikChange II was utilized to introduce the FLAG epitope and point mutations in FLAG-ChEL in the pCMS-EGFP vector. QuikChange Lightning was used to introduce point mutations in mouse TG in the pcDNA3.1 vector. PCR was carried out with a 2710 Thermal Cycler (Applied Biosystems). PCR products were treated with DPN I enzyme and transformed according to the manufacturer's instructions. For each mutagenesis, four plasmids were purified and subjected to Sanger sequencing (using the following primers: 5'-cacctccacaatcaatgaagac-3'; 5'-ggcttgaagcaattgaagac-3'; 5'-ggagccaaggatgcacagta-3'; and N-terminal). Plasmid clones of interest were amplified in DH5 α -competent cells and re-sequenced in the mutation zone.

Thyroglobulin dimerization promotes T_3 formation

PCCL3 cell culture

A disruption of *Tgn* gene expression in PCCL3 rat thyrocytes was created via a stable CRISPR-Cas9-mediated homozygous 138-base pair deletion in the PCCL3-clone 7F that included 65 base pairs of the *Tgn* intron 3 and 73 base pairs of the *Tgn* exon 3, confirmed by genomic DNA sequencing. Both clone 7F and control PCCL3 cells were cultured in DMEM/F-12 supplemented with 5% FBS plus penicillin/streptomycin and a four-hormone mixture containing 1 μ g/ml of insulin, 1 nM hydrocortisone, 5 μ g/ml of apo-transferrin, and 1 milliunit/ml of TSH.

For stable expression of bovine TG or mouse TG, PCCL3-clone 7F cells were seeded at 300,000 cells/well in 6-well plates, and 24 h later were transfected (using Viafect) with either a pCB7 plasmid (bearing a hygromycin resistance gene) and encoding WT bovine TG, or co-transfected with a pcDNA3.1 vector encoding WT mouse TG plus a linearized hygromycin-resistance vector (at a 20:1 ratio, respectively). A pool of PCCL3 cells resistant to hygromycin at 400 (for bTG) or 200 μ g/ml (for mTG) were selected and grown for 4 weeks before trypsinizing and mixing the cells to homogeneity, and seeding in 24-well plates. After 24 h, culture medium was replaced with serum-free medium with or without TSH for an additional 24 h; cells without TSH were then cultured for an additional 4 days. Bathing media were collected and the cells were lysed in RIPA buffer containing protease inhibitor mixture. Both cell lysates and media were pre-cleared by centrifugation.

Transient transfection and immunoprecipitation

293T cells were cultured in DMEM with 10% fetal bovine serum at 37 °C in a humidified 5% CO₂ incubator. 293T cells were seeded at 50,000 cells/well in 24-well plates 24 h prior to transfection. 500 ng of plasmid DNA per well was transiently transfected using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. At 24 h after transfection, the cells were washed with PBS and re-fed with serum-free media for another 24 h. Bathing media were collected and the cells were lysed in RIPA buffer containing protease inhibitor mixture and processed as for stably transfected cells (see below).

For studies related to iodination of secretory FLAG-ChEL, the media was incubated with anti-TG Ab overnight at 4 °C, and the immunoprecipitates were recovered with protein A-agarose. Immunoprecipitates were washed three times with nondenaturing IP buffer (25 mM Tris, pH 7.4, 0.1% Triton X-100, 5 mM EDTA, 0.1 M NaCl) and twice with PBS, pH 7.4.

For secretory FLAG-ChEL co-precipitation with TG, samples were immunoprecipitated with anti-FLAG M2-agarose gel, washed, boiled in 1% SDS, diluted 10-fold into immunoprecipitation buffer lacking SDS (25 mM Tris, pH 7.4, 0.1% Triton X-100, 5 mM EDTA, and 0.1 M NaCl), and re-immunoprecipitated with anti-FLAG M2-agarose gel for analysis by immunoblotting with anti-TG.

In vitro enzymatic iodination of TG

Iodination *in vitro* was performed as recently described (5), including lactoperoxidase (30 ng/ μ l), glucose (2 μ g/ μ l), glucose oxidase (0.352 ng/ μ l), 100 μ M NaI, and 50–150 ng/ μ l of protein

from bathing media containing secreted TG secretion, or from immunoprecipitates of secreted FLAG-ChEL. Incubations were initiated with the addition of glucose oxidase, incubated for 2 h at 37 °C, and stopped by addition of gel sample buffer and boiling for 5 min.

Western blotting

Samples were subjected to SDS-PAGE under either reducing or nonreducing conditions, electrotransfer to nitrocellulose, and blocking, as described previously (5). Primary mouse mAb anti- T_3 was diluted at 1:1000 containing 500 ng/ml of free T_4 (to eliminate any possibility of T_4 cross-reactivity) and incubated overnight at 4 °C. In parallel immunoblots, primary rabbit polyclonal anti-TG was diluted 1:5000 in 5% BSA/TBS-T and incubated at room temperature for 1 h. Primary mouse mAb anti-FLAG was diluted at 1:1000 and incubated overnight at 4 °C. 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 Western-Bright Sirius kit (Advansta) and digital images were captured in a Fotodyne work station; exposure times averaged 20 s for anti- T_3 blots, 5 s for anti-TG blots, and 30 s for anti-FLAG blots.

Quantitation of T_3 /TG or T_3 /FLAG-ChEL band intensity ratio

Band intensities were quantified using ImageQuant 5.2 (Molecular Dynamics) as previously described (5). The intensities of the bands corresponding to T_3 immunoreactivity within the TG or ChEL band were compared with the direct TG or ChEL immunoreactivity of the same band using anti-TG or anti-FLAG immunoblotting. The ratio of band intensities was set to a control value of 1.0 from which experimental samples were compared.

Data analysis

Statistical analyses were done utilizing unpaired Student's *t* test with two-tailed *p* value (Figs. 1D, 6, A and B, and 7C) or by one-way analysis of variance followed by Dunnett's test (Figs. 2D and 3D). Statistical values were calculated with GraphPad Prism version 6. The level of significance for all statistical tests was set to *p* < 0.05. Data are presented as mean \pm S.D.

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Relationship between the dimerization of thyroglobulin and its ability to form triiodothyronine

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