New insights in the human thyroglobulin structure

van de Graaf, S.A.R.

Citation for published version (APA):
Human thyroglobulin can be expressed, glycosylated and iodinated in rabbit reticulocyte lysate.

SAR van de Graaf, GJM Veenboer, JJM de Vijlder, C Ris-Stalpers.

**ABSTRACT**

An *in vitro* strategy to study the structure-function relationship of human thyroglobulin (TG) is described. It is based on transcription and translation of a 1726 bp 5' TG cDNA fragment that is suitable for expression in rabbit reticulocyte lysate (RRL). The TG protein expressed can be glycosylated by adding canine pancreas microsomal membranes and subsequently iodinated in the same aliquot. The addition of microsomal membranes results in an apparent shift in molecular weight on SDS PAGE of the TG protein expressed. This shift reflects glycosylation since it can be reversed by the addition of a deglycosylating enzyme. We have previously described a homozygous G to A change on position 229 of the TG cDNA in three hypothyroid patients with the clinical phenotype of a TG synthesis defect. The amino acid change resulting from this mutation is located in a putative N-glycosylation site on amino acid position 57. We prepared an expression construct harboring this mutation and after expression no difference in the glycosylation induced SDS PAGE profile was detected between the nt G229 and nt A229 expression construct variants. SDS PAGE was also used to analyze the combined translation / iodination assay reactions. Under the used conditions the native N-terminal human TG protein was not iodinated whereas the glycosylated TG was. The described strategy of a combined glycosylation and iodination assay in RRL is a promising basis for *in vitro* structure-function analysis of wild type and mutated human thyroglobulin.
INTRODUCTION

Thyroglobulin (TG) is the major iodoglycoprotein of the thyroid gland and is responsible for the synthesis, storage and secretion of thyroid hormone. After initial transcription of the TG gene with an open reading frame of 8307 base pairs, subsequent translation of the mRNA leads to a TG precursor molecule of 2768 amino acid residues (1). The post-translational route of TG starts with the signal peptide, encoded by 19 N-terminal amino acid residues, that directs the uptake in the endoplasmic reticulum and is cleaved off. Here, the first mannose and glucose residues are added and further glycosylation takes place in the Golgi apparatus. Based on the presence of the N-glycosylation consensus sequence N-X-S/T, 20 putative N-glycosylation sites are present along the amino acid sequence of human thyroglobulin. Of these 20, in vivo asparagine residues 91, 477, 1850 and 2103 escape glycosylation (2). Glycosylation of TG influences numerous post-translational processes, varying from interaction of TG with thyroid microsomes (3), targeting of the macromolecule to subcellular and extracellular compartments (4) and antigenicity of TG (5) to binding of molecular chaperones (6). In addition, glycosylation is known to influence TG structure (7) and to promote thyroid hormonogenesis (8,9,10). We previously identified a variation in the N-glycosylation consensus site at amino acid position number 57: glycine is changed to serine in the asparagine/glycine/serine motif in three patients with hypothyroidism due to a putative TG synthesis defect (11). Although the consensus for putative glycosylation is not changed, we investigated with SDS PAGE whether glycosylation of an N-terminal TG fragment is influenced by the G58S change.

In vivo TG molecules contain 10% carbohydrate structures and have a molecular weight of 330,000. Upon routing through the thyrocyte cell, homodimers are formed which are stored in the lumen of the thyrocyte follicles (12). Catalyzed by the apical membrane-associated thyroid peroxidase and in the presence of a \( \text{H}_2\text{O}_2 \) generating system, specific tyrosine residues within the TG molecule are iodinated and subsequently coupled under oxidative conditions to form thyroid hormone (13,14,15). Theoretically all 66 tyrosine residues along the sequence can be iodinated but only 3-17 are identified as iodotyrosyl residues in vivo. Seven of these have been reported to be involved in thyroid hormonogenesis because of accepting or donating an iodophenylgroup in the 'coupling reaction' (12,16). We previously identified a nonsense mutation inducing a premature stop codon in a family with mild hypothyroidism and goiter that results in a truncated protein of 277 amino acids. This
protein fragment contains the tyrosine residues at positions 5 and 130 implicated important in thyroid hormonogenesis (17). Since this protein fragment results in mild hypothyroidism these two sites seem adequate to synthesize thyroid hormone, when iodine intake is sufficient. A similar TG fragment of 295 amino acid residues, as identified in the Dutch goats strain (18,19) is still functional in vivo as well. Furthermore, a human TG fragment of 200 amino acid residues produced in a baculovirus expression system is capable to produce thyroid hormone in an in vitro iodination assay (20).

In order to study the structure-function relationship of TG in thyroid hormone synthesis two sources have been described: TG isolated from thyroid tissue and TG protein fragments synthesized in an eukaryotic baculovirus expression system (20). Both sources have major drawbacks that influence a subsequent functional assay: TG isolated from thyroid tissue is heterogeneous iodinated and the baculovirus system has, compared to a mammalian cell-based system, limited ability to terminally glycosylate glycoproteins (21). When the generally accepted in vitro iodination and coupling method was used on a TG fragment of 200 amino acid residues originated from the baculovirus expression system, the method was unable to distinguish between thyroid hormone synthesis capacity of 'wild type' TG (containing seven tyrosine residues) and of 'variant' TG (tyrosine residues substituted by phenylalanine)(22). The rabbit reticulocyte lysate expression system is promoted as an alternative for eukaryotic in vitro translations and provides TG protein that is not iodinated and processing events such as signal peptide cleavage and glycosylation occur when microsomal membranes are present during translation. In this system we studied both glycosylation and iodination of human TG. A wild type TG expression construct encoding 577 N-terminal amino acid residues of human TG including the signal peptide, 14 tyrosine residues, and 6 putative N-glycosylation sites was used.

**MATERIAL AND METHODS**

**Expression constructs**

All subcloning strategies are schematically shown in Figure 1 resulting in 4 expression constructs: TG 577/G58, TG 577/S58, TG 295/G58, and TG 295/S58. The wild type fragment (TG 577, encoding amino acids -19 through 558) was recovered from the previously described TGI expression construct (23) with EcoR I (Biolabs) digestion, containing a small part of extra polylinker sequence and the TG cDNA sequence -6 through 1726. The mutant fragment (TG 295, encoding amino acids -19 through
276) was recovered via the same strategy from the previously described TG-M expression construct (17). Both fragments were subcloned into the EcoR I (Biolabs) linearized pcDNA3.1 (-) Myc/His B expression vector (Invitrogen) using standard methods.

In both the TG 577 and the TG 295 construct a G to A mutation was made on cDNA position 229. RT-PCR on thyroid RNA of the previously described patient 5 (11) with this specific mutation was done using forward primer TGI s (23), reverse primer 3R (11) and Expand High Fidelity Enzyme (Boehringer Mannheim). The resulting 1356 bp fragment was digested with PflM I (Biolabs) and the 478 bp fragment, containing the homozygous nt A229 variation was isolated. This fragment was exchanged with the wild type fragment containing nt G229. The presence of the nt 229 variation was checked by automatic sequencing as described in (23).

![Diagram of TG expression constructs](image)

Figure 1 Schematic presentation of TG expression constructs. From both the TG 577 and the TG 295 construct some cDNA (relevant nt positions on top) and corresponding protein characteristics (relevant aa positions in italics) are shown. Extra nt sequences from polylinker sequence of pcDNA 3 (23) is given. The termination codon (Term) and the bovine growth hormone polyadenylation signal (BGH pA) are indicated. Of both expression plasmids a construct with either a G or an A on nucleotide position 229 has been constructed leading to an aa variation at position 58.
Coupled transcription/translation assay

From the TnT T7 Coupled Reticulocyte Lysate System (Promega) kit 25 µl rabbit reticulocyte lysate (RRL), 1 µl reaction buffer, 1 µl T7 RNA polymerase and 2 µl amino acid mixture lacking cysteine were mixed, and 1 µl Rnasin Ribonuclease Inhibitor (Promega), 3 µl mixture of 35S-methionine and 35S-cysteine (ICN Pharmaceutics; Tran35S-label), 300 ng DNA (TG 577/G58 or TG 577/S58 or TG 295/G58 or TG 295/S58) and H2O were added to a total volume of 50 µl and incubated at 30°C for 90 min. Addition of Canine Pancreatic Microsomal Membranes (CPMM) (Promega) to obtain glycosylation caused decreased expression, so RRL reactions were upscaled 5 times. Both the recommended 4.8 µl CPMM (+) per 50 µl RRL reaction and 0.96 µl CPMM (+) were used. 35S-label, used for visualization of the synthesized protein, was replaced by H2O in case of subsequent radioactive iodination assay. Total protein concentration in RRL is 200 mg/ml. Iodination assay In order to standardize reaction conditions, 120 µl RRL, 4 µl reaction buffer and 116 µl H2O were added to 10 µl of - CPMM RRL reactions. To 250 µl of translation RRL reaction (- CPMM and + CPMM), 5 µl glucose, 5 µl lactoperoxidase (Sigma) and 5 µl glucose oxidase (Sigma) were added. 125I (Amersham) in a final concentration of 43 nM was added to the non-radioactive translation RRL reactions only, because the 35S label overruled the 125I label on SDS PAGE scan and in counting. After addition of 0.4 M NaH2PO4/Na2HPO4 pH 7.0 to a total volume of 300 µl, the samples were incubated at 37°C for 30 min and subsequently terminated with 60 µl stopsolution (final concentration: 0.25 M methimazol, 10 mM NaI and 0.1 mM sodiumazide). Immunoprecipitation Before SDS PAGE, the TG protein products from 360 µl translation and iodination RRL reactions were immunoprecipitated using a rabbit polyclonal antibody specific for human TG (24) and coupled to Protein A-Sepharose CL-4B (Pharmacia Biotech).

Deglycosylation

After the coupled transcription/translation assay using TG 577/G58 as template (50 µl RRL reaction - CPMM and 100 µl RRL reaction + CPMM), the samples were denatured. Subsequently the supernatant of the sample + CPMM was split and one sample was treated with PNGase F enzyme (Biolabs) according the manufacturer’s protocol.

SDS PAGE

RRL samples were run on a 12.5 % SDS-polyacrylamid gel according to Laemmlli with a general Mol wt marker (Biolabs, Rainbow). Gels were dried afterwards. The radioactive signal of expressed proteins was detected using a Phosphorimager and
Image Quant software (Molecular Dynamics). \(^{125}\text{I}\) label was also counted from gelslices in cpm (Christal II (Packard)).

**RESULTS**

For *in vitro* translation purposes a basic 1726 bp human TG cDNA fragment was inserted downstream from the T7 RNA polymerase binding site in the expression vector pcDNA3.1. Coupled transcription/translation in a rabbit reticulocyte lysate (RRL) expression system of this construct results in 577 amino acid residues of TG (-19 through 558), including 14 tyrosine residues and 6 putative N-glycosylation sites (Fig 1). A smaller N-terminal TG protein fragment was made by introducing a premature stop codon at nt 886 in the basic 1726 bp expression construct. From both the wild type and mutant constructs a G to A variant was made on position 229, resulting in a glycine to serine change at amino acid position 58 after translation. Glycosylation of the expressed protein product was achieved by adding canine pancreatic microsomal membranes (CPMM) to the transcription / translation reaction. Figure 2 depicts the SDS PAGE scan showing the effect of CPMM addition to the RRL. This results in a shift in apparent molecular weight of the native TG protein product (64 kDa, lane 2) to 80 kDa (lane3). To validate the presence of carbohydrate structures on the 80 kDa TG protein product, this sample was deglycosylated by PGNase F (lane 4). The deglycosylated product shows a profile identical to the native TG protein product.

![Figure 2 Glycosylation and deglycosylation of TG 577. SDS PAGE gel scan of \(^{35}\text{S}\) methionine labeled TG protein (TG 577/G58), expressed in the absence (lane 2) and presence (lanes 3 and 4) of microsomal membranes (CPMM). Lane 4 shows the PGNase F treated sample from lane 2. Marker band of 75 kDa apparent Mol wt is indicated. Closed arrow points to native protein and open arrow points to glycoprotein.](image)
In three hypothyroid patients with a putative TG synthesis defect, we detected a homozygous variation on nt position 229 resulting in an aa variation on position 58 (glycine → serine). Because of its location in the putative N57-glycosylation site, we studied the ability of this site to bear carbohydrate structures when position 58 was varied. All four constructs as shown in Figure 1 were translated in the presence or absence of CPMM and analyzed on SDS PAGE (Fig 3). The TG 577 construct generates a native TG protein of 64 kDa with 6 putative N-glycosylation sites while the TG 295 construct generates a TG protein of 30 kDa with 3 putative N-glycosylation sites (lanes 1 and 2). Comparing the glycoprotein profiles of G58 and S588 variations (lanes 3 and 4), no difference was detected in apparent Mol wt implying that the change from glycine to serine on position 58 does not alter the glycosylation pattern of the molecule.

The native and glycoproteins from Figures 2 and 3 were validated to be TG by immunoprecipitation with a polyclonal rabbit-anti-human-TG antibody and subsequent analysis on SDS PAGE (data not shown).

![Figure 3 Glycosylation of TG proteins with aa variation in N57-glycosylation site. Gel scans of SDS PAGE of TG protein containing 6 putative N-glycosylation sites (TG 577) and TG protein containing 3 putative N-glycosylation sites (TG 295) expressed in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of microsomal membranes (CPMM). Marker bands of 30, 35 and 75 kDa are indicated. Closed arrow points to native TG protein and open arrow points to TG glycoprotein.](image)

After coupled transcription / translation in RRL of the TG 577 expression construct, iodination was assayed in the same aliquot. TG protein fragments were immunoprecipitated after the combined assays and analyzed on SDS PAGE. Proteins were visualized using either $^{35}$S in the translation assay or $^{125}$I in the iodination assay.
**Figure 4 Combined translation and iodination of TG protein.**

Gel scan of SDS PAGE of coupled transcription/translation reactions of TG 577/G58 construct (lanes 4-6) in the presence of \(^{35}\)S labeled methionine. Iodination reactions of the same reactions in the presence of \(^{125}\)I (lanes 7-9). Identical reactions without DNA template shown in lanes 1-3 (transcription/translation) and lanes 10-12 (iodination). Gelslices, location and size indicated by A, B and C, are counted in cpm of \(^{125}\)I. Marker band of 75 kDa, native protein (closed arrow), glycoprotein (open arrow) and iodoglycoprotein (grey arrow) are indicated.

The gel scan (Fig 4) shows the TG protein input into the iodination assay (lanes 4, 5 and 6). The addition of the recommended amount of microsomal membranes drastically reduced the yield of the reaction and a lower amount improves the yield, but does not accomplish full glycosylation. Iodinated TG protein (lanes 7-9) is only detectable when glycosylation conditions during prior translation are optimal (lane 9) and the iodoglycoprotein product had an apparent Mol wt of 70 kDa. From the gel three slices of each lane with \(^{125}\)I labeled TG protein were counted: representing TG glycoprotein (80 kDa, A), TG iodoglycoprotein (64-70 kDa, B) and background (C). The background \(^{125}\)I signal (lanes 7-9 slices C and lanes 10-12 slices A, B, C) varied between 4 and 18 cpm. Lane 9 shows a clearly distinct 50 cpm count in slice B,
corresponding to the iodinated glycosylated form of TG. There is no protein product observed at this position in the samples that are not glycosylated (lane 7). There is also no protein product of this apparent Mol wt in the glycosylated sample that has not been subjected to the iodination reaction (lane 6).

**DISCUSSION AND CONCLUSIONS**

Previously reported studies have shown that non-mammalian glycosylation and too optimal iodination conditions hamper *in vitro* thyroglobulin iodination studies. To overcome these issues we combined *in vitro* coupled transcription / translation, glycosylation and iodination assays in rabbit reticulocyte lysate. The rabbit reticulocyte lysate expression system was chosen as a source for TG protein because in this system mammal glycosylation can be conditioned by the presence of canine pancreatic microsomal membranes and additionally the product has no iodothyrosine residues. To detect the expressed protein, a radioactive amino acid mixture is added to the incubation (\(^{35}\)S Met/Cys).

As a basis the 5' TG cDNA fragment of 1726 bp was subcloned in an expression vector with an upstream T7 polymerase start site. Translation of this construct results in an N-terminal TG fragment of 577 amino acids including the 19 amino acid signal peptide, 6 putative N-glycosylation sites and 7 tyrosine residues that are accessible for iodination (including tyrosines 5 and 130). From both a human and an animal model it is known that *in vivo* TG fragments of even a smaller size (~ 35 kDa Mol wt) are able to synthesize thyroid hormone under adequate iodine intake (17,25). The addition of microsomal membranes induced the expression of a TG glycoprotein. The gel shift on SDS PAGE due to glycosylation of the TG protein was used to study the nt 229 variation, which results in a G58S change upon translation. Although this change does not abolish the N-X-S/T glycosylation consensus on site N57 (11), it is possible that the change from the nonpolar glycine residue to the polar serine residue alters the accessibility of this site for the glycosylating enzymes. No difference in gel shift was detected comparing G58 and S58 variants of either a TG protein with 6 or with 3 putative N-glycosylation sites (Fig 3, TG 577 and TG 295, respectively). It is however possible that a change in at this site is not reflected in the apparent Mol wt on SDS PAGE. It can also be that nt 229 and aa 58 variation results in a change in the TG protein structure i.e. the folding instead of the glycosylation pattern. An endoplasmic reticulum storage defect could be one of the expected results (26) and to investigate this, thyroid tissue of the patients concerned should be analyzed histologically. Based on our studies we do not expect an obvious role for the
homozygous variation in the TG cDNA in the patients with mild hypothyroidism as previously described (11). It is however striking that the nucleotide substitution was only homozygous present in three patients with mild hypothyroidism due to a clinically defined TG synthesis defect, and not in 34 control alleles (1,10).

Both the translation and iodination assays were done in the presence of radioactive label, making it possible to visualize a relative low amount of expressed protein. We aimed to achieve suboptimal iodination conditions compared to previous studies by leaving the newly synthesized TG in the protein rich RRL mixture during the iodination reaction. The iodide concentration in our iodination assay was approximately 13pmol I⁻ / 25 mg total protein, in the same range as the concentration in the normal thyroid gland estimated to be 50 pmol I⁻ / 25 mg total protein (27, 28).

After combining the RRL expression system with the iodination assay, followed by immunoprecipitation to isolate the TG protein fragment it was concluded that only TG protein synthesized in the presence of microsomal membranes was iodinated.

This implies that glycosylation is a prerequisite for iodination of TG when the iodide concentration is in the physiological range (Fig 4).

The gel scan in Figure 4 is representative for 3 different experiments using the same batch of rabbit reticulocyte lysate and microsomal membranes, which is important because differences in levels of expression and glycosylation were observed between different batches.

The strategy we describe here of combined translation, glycosylation and iodination in rabbit reticulocyte lysate has the potential to be used for future structure-function analysis of wild type and mutated human TG.
REFERENCES

1. van de Graaf SAR, Ris-Stalpers C, Pauws E, Mendive FM, Targovnik HM, de Vijlder JJM 2000 Up-to-date with the human thyroglobulin sequence. submitted


24. den Hartog MT, de Boer M, Veenboer GJM, de Vijlder JJM 1990 Generation and characterization of monoclonal antibodies directed against noniodinated and iodinated thyroglobulin, among which are antibodies against hormonogenic sites. Endocrinol 127:3160-3165.


