New insights in the human thyroglobulin structure
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Chapter 8

GENERAL DISCUSSION
Heterogeneity of TG

**DNA sequence**

The revisions and polymorphisms in the TG cDNA sequence and their effect on protein sequence have been described in Chapter 2. The 15 polymorphic nucleotides positions, 14 elucidated from a group of Caucasian individuals and an additional one in a Japanese population, are of importance for TG DNA analysis. For instance, Restriction Fragment Length Polymorphism analysis that can be used in linkage studies is influenced when a polymorphism changes the restriction enzyme recognition sequence in a fragment of interest. Location of sequence polymorphisms is also important to prevent misinterpretation in mutation analysis by fast screening techniques as Single Strand Conformational Polymorphism, Denaturing Gradient Gel Electrophoresis and Denaturing High Performance Liquid Chromatography.

**RNA transcripts**

A heterogeneous pool of TG transcripts exists in thyroid tissue. This heterogeneity is the result 1) of polymorphisms, when DNA sequences are different on both alleles, 2) of alternative splice events (Chapters 2 and 4), and 3) of the differential use of polyadenylation cleavage sites (Chapter 3). The ratios of normal and alternative splice transcripts and presumably also the use of different polyadenylation cleavage sites vary in thyroid tissue of both healthy individuals and patients. In designing primers for RT-PCR amplification, often a step prior to mutation analysis, one has to take this heterogeneity into account to minimize misinterpretation of absent amplification.

**Protein structure**

During maturation of TG polypeptides, described in Chapter 1 (Fig 2), various post-translational processes take place. These include glycosylation and dimer conformation (initiated in the endoplasmic reticulum), sulphation and phosphorylation (probably during vesicular transport to the apical membrane), additional sialylation (at the apical membrane), iodination of specific tyrosine residues and coupling of iodotyrosyl residues to thyroid hormones (at the border of the apical membrane and the follicular lumen). This maturation of the TG protein is guided by strong quality control. However this control does not prevent microheterogeneity of TG. Microheterogeneity in glycosylation, conformation, sialic acid content, iodination degree and thyroid hormone content is present in the follicular lumen of every individual. This may in part be caused by small alterations in the TG polypeptide chains that result from translation of heterozygous polymorphic nucleotides. Ten amino acid positions have a polymorphic character and although none of them is positioned in an N-glycosylation consensus sequence or concerns a tyrosine residue,
post-translational effects cannot be ruled out. *In vitro* translation of TG mRNA isolated from goitrous thyroid tissue of Afrikaander cattle, containing a transcript carrying a nonsense mutation and a prominent alternative splice transcript, yielded two TG polypeptides different in length (1). This suggests that alternative splice transcripts can result in TG polypeptides *in vivo* and thus alternative splice events may also contribute to the microheterogeneity of the follicular TG.

**Role of TG detection**

**TG in primary congenital hypothyroidism**
The TG polypeptide pool in the follicular lumen shows microheterogeneity as mentioned in the preceding section. Leakage from this pool to the circulation can occur via the tight junctions, via the megalin-mediated transcytosis and via the phagolysosomal pathway. Therefore, a heterogeneous population of TG molecules can be expected to circulate in the blood of healthy individuals.

In general it is assumed that TG plasma levels reflect thyroidal TG levels and rate of TG synthesis. In patients without a thyroid gland, agenesis, the absence of plasma TG is a strong indication for diagnosis. Vice versa, high levels of plasma TG are indicative for a stimulated thyroid gland as is demonstrated in patients with a total iodide organization defect caused by mutations in the TPO gene and in patients with an iodide trapping defect caused by mutations in the NIS gene. Persistent stimulation of the thyroid gland by TSH has been reported to alter the tight junction structure and subsequently to increase TG leakage from the follicular lumen. Despite the TSH stimulation in hypothyroid patients with a TG synthesis defect, plasma TG levels are described to be undetectable or low. There are several reasons for this feature: the TG mRNA can be instable as described for the Dutch goats and the turnover of the mutated TG polypeptides can be high as a result of quality control and high TSH pressure. It can however also be assumed that aberrant TG molecules are retained in the thyroid gland as a result of the thyroidal quality control process. If this is the case plasma TG levels will not reflect thyroidal TG levels or rate of synthesis. For this reason the distinction often made between a qualitative and a quantitative TG synthesis defect does not shed any light on the underlying molecular cause, but largely represents the activity of the quality control process for particular TG molecules. Moreover, it is always possible that a mutation influences the tertiary structure to such a degree that one or more epitopes for the TG antibodies are lost, decreasing the TG concentration determined using anti-TG antibodies.
TG in autoimmune diseases
The thymus is the central organ for induction of T-cell self-tolerance that results from presentation of autoantigens within the thymus. Although TG is reported to be expressed in the normal thymus (2), TG antigenicity seems relevant to autoimmune thyroid disease. Injection of TG into animals induces a thyroiditis and is proposed as a model for human autoimmune thyroiditis (3,4). The degree and location of iodination in TG influence this response. For example, autoimmune thyroiditis was induced in mice by a 12-amino acid residue peptide containing T_4 at tyrosine residue 2554 of human TG, but not by the same peptide without T_4 (4). Experimental autoimmune thyroiditis was reported to be suppressed by oral administration of human TG (5,6). Monoclonal antibodies recognizing various TG epitopes associated with iodothyronines, iodotyrosines, and specific tertiary structures have been described (7,8).

In humans, autoantibodies occur in healthy individuals as well as in patients with autoimmune thyroid diseases, but may recognize different epitopes (9). Epitope analysis provides information about the tertiary structure of TG and can be helpful in the analysis of autoantibodies to TG. The information from epitope analysis can also give clues to the liability of the immunogenic assays used to determine TG concentration in blood. However, the source of TG (from cytosol or follicular lumen, from normal or pathological tissue or blood) used for epitope analysis should be well defined to avoid misinterpretation.

TG as marker
TG is clinically relevant in the assessment of differentiated thyroid cancer after thyroidectomy. Patients with thyroid cancer are post-surgically monitored for disease recurrence by measurement of plasma TG and 131-I whole body scan (10). In case of a partial thyroidectomy, one way to discriminate in the origin of plasma TG is the reactivity to lectins reflecting the difference in carbohydrate chain composition on TG produced by carcinoma cells and normal thyroid tissue (11). While immunoreactive TG in plasma is a widely used marker, the TG antigen used for antibody production has received suprisingly little attention. Antibodies have usually been raised to TG isolated from uncharacterized goiters or from thyroids obtained from patients with Graves' disease. TG in thyroid cancer is typically low in iodine content and several studies have suggested additional structural differences in glycosylation and peptide structure (12,13).

Moreover, interfering autoantibodies are a major limitation to the current use of TG-immunoassays and, although performing an additional thyroglobulin recovery test
proves to overcome this problem (14), development of a principally different assay seems favorable. A promising assay for this purpose is RT-PCR as it can be used to detect circulating cancer cells in the peripheral blood of patients with malignancies (15). An RT-PCR assay set up to measure TG mRNA from circulating thyroid cells in the peripheral blood of healthy individuals showed accurate and quantitative detection of TG mRNA and may provide a useful tool in monitoring patients with thyroid cancer post-surgically (16). The amplification primers used in this assay are located in the third and fourth exon that is known to be deleted in one of the alternatively spliced TG mRNA transcripts (Chapter 2). Especially in the case of monitoring for recurrence of thyroid cancer post-surgically, misinterpretation of absent amplification can have severe clinical consequences. Therefore, designing primers located outside an alternative splice region, for instance covering exons one and two, is recommended.

**TG synthesis defect**

*Mutation analysis*

Polymorphisms in the coding region are randomly dispersed in the 48 exons and the size of these exons varies from 63 bp (exon 35) to 1101 bp (exon 9)(Chapter 2). In order to quickly screen for mutations several techniques are available: Single Strand Conformational Polymorphism (SSCP) analysis and Denaturing Gradient Gel Electrophoresis (DGGE), these are techniques that are rather time consuming and expensive. Recently the Denaturing High Performance Liquid Chromatography (DHPLC) analysis has been developed, an innovative and cost-efficient technique that enables a large degree of automation (17). After these screening methods, DNA fragments that show an aberrant profile possibly reflecting a mutation have to be sequenced.

We initially started to use SSCP, but since this resulted in so many aberrant profiles reflecting the many polymorphisms and alternatively spliced products we opted for direct sequencing of all RT-PCR amplified fragments (Chapters 4 and 5). Seven hypothyroid patients with a putative TG synthesis defect were screened for mutations in TG cDNA by RT-PCR amplification of 500 bp overlapping fragments on RNA isolated from thyroid tissue and subsequent nucleotide sequence analysis. In one patient a homozygous nonsense mutation was found resulting in a truncated TG molecule after translation and this mutation co-segregated with the clinical phenotype in the family (Chapter 5). Although in one of the other families described in Chapter 4 autosomal dominant inheritance of the clinical presentation indicating a
TG synthesis defect was reported (18), our studies did not show any mutation in TG. Also, in the other five patients, no relation was found between TG cDNA sequence variations and the disease (Chapter 4).

In three of the six patients with a wildtype TG cDNA sequence, some debate is possible on the adenosine / guanidine variation located on nucleotide position 229. As this nucleotide variant was detected homozygously in three patients and not in any of the 40 healthy alleles, it may be a mutation. Family screening could have given more information but unfortunately no additional material was available. After translation, the variation results in a serine instead of a glutamic acid residue on position 58 located within the three amino acid residue N-glycosylation consensus sequence of asparagine residue 57. Although this is a conservative amino acid change, it cannot be ruled out that the glycosylation potential of amino acid 57 was changed. After in vitro translation of a TG fragment containing either a serine or a glutamic acid on position 58, the electrophoretic mobility shift due to addition of carbohydrate structures was unaltered. While a functional link for the variation has not been established (yet), nucleotide position 229 is currently considered to be polymorphic.

Since 1991, five defects in the human TG mRNA have been identified that are linked to primary congenital hypothyroidism (Chapter 1,Chapter 2). Two of the mutations create an early termination codon (19,20). They occur in a CpG dinucleotide that, through methylation of cytosine and subsequent deamination, is mutated to thymine (21). Both mutations concern the CGA codon encoding for an arginine residue, that is generally considered a 'hot spot' for mutations (22). In both cases a truncated TG polypeptide is the result of translation but in one case this is partially prevented by upregulation of an alternative splice transcript also seen in thyroid tissue of healthy individuals. It is suggested that this deleted transcript is translated and partially recovers thyroid hormonogenesis.

Deletions of exon 4 and exon 30 have been reported (23,24). In case of the exon 4 deletion, the cause is a mutation in the intron splice site. Exon 4 is important for TG function because it contains the tyrosine residue 130 involved in iodination and coupling reactions and a cysteine rich repeated type 1 domain. However, the importance for TG function of exon 30, containing part of a cysteine rich repeated domain type 3, is less clear.

Clinical description
Goiter development was one of the classical characteristics in the clinical presentation of hypothyroidism. In iodine deficient areas goiter develops, often even before birth,
but can be prevented or treated by iodine administration. Goiter development as a result of inborn errors in thyroid metabolism is largely prevented by early treatment with thyroid hormone, that is possible because of the neonatal screening on congenital hypothyroidism. The patients with an identified TG gene mutation (Chapter 2) all developed goiter because neonatal screening was not operational. For patients with a putative TG synthesis defect, isolation of TG protein from thyroid tissue and determination of sedimentation coefficients and electrophoretic parameters has often been used for diagnosis. Because of the microheterogeneity of TG in the cytosol and in the follicular lumen, one can question the value of TG protein isolation and subsequent biochemical analysis for diagnosing a TG synthesis defect. Immunohistological staining of TG can give more information because cytosolic storage can implicate arrest of TG maturation by the quality control. However, it does not indicate whether this arrest is caused by a defect in TG or in other proteins important for TG maturation. Furthermore, using light microscopy, it is difficult to distinguish between TG arrest in the ER and Golgi compartments and TG arrest in the endocytotic vesicles. In 4 of the 8 cases with an identified mutation in the TG gene, the majority of TG is immunologically present in the cytosol while only low levels of TG were present in the follicular lumen and in blood plasma. Mutation analysis in the TG gene is not hindered anymore by the scarcity of removed thyroid tissue, because the human TG gene structure is elucidated facilitating the use of genomic DNA as template in PCR amplification. Also, RT-PCR amplification can be performed nowadays on RNA isolated from circulating thyroid cells (16). A point of caution is that it is not known whether the whole TG mRNA can be recovered these circulating cells, or that only certain splice variants are present. Apart from the low or undetectable plasma TG concentrations in combination with high plasma TSH level and goiter (in the past) several other diagnostic characteristics are indicative for a TG synthesis defect. As expected from the high TSH plasma level, the uptake of RAI is rapid and high. Since the iodination mechanism is intact most of the iodide taken up will be oxidized and bound to TG protein. Subsequent blocking of the inward iodide transport by sodiumperchlorate administration results generally in a discharge below 20% of the administered RAI. However, partial discharge, often seen in patients with a clinically diagnosed partial iodide organification defect, is sometimes seen in patients with a putative TG synthesis defect. This can be caused by inadequate iodide binding capacity of TG molecules with an aberrant structure. This is in fact observed in a patient with a TG synthesis defect in which a point mutation is detected in exon 17 (25).
Another aspect related to a TG synthesis defect is the occurrence of low molecular weight iodinated material (LOMWIOM) in urine. This phenomenon was first seen in the Dutch goats (26). It has been used as a diagnostic criterion in patients with a putative TG synthesis defect (27), but although three of the six patients we studied contained LOMWIOM in their urine, they had no mutation in the TG gene (Chapter 4). Furthermore, in another study the presence of LOMWIOM in urine has been determined in different CH patients (unpublished report). LOMWIOM was observed in 19 out of 20 patients with a putative TG synthesis defect, in 1 out of 9 patients with a dislocated thyroid gland and in 3 out of 4 patients with a partial iodide organification defect. No LOMWIOM was observed in 2 patients with a total organification defect and in 2 patients with Pendred's syndrome.

Some authors make the distinction between a quantitative and qualitative TG synthesis defect, often based on the response of plasma TG levels on stimulation with bovine TSH (28,29). When plasma TG levels are very low and do not increase upon TSH administration the TG synthesis defect is termed quantitative. A qualitative TG synthesis defect describes a plasma TG level responsive to bovine TSH. The distinction between qualitative and quantitative is difficult to maintain since it is not excluded that a qualitative defect in TG synthesis results in aberrant TG molecules which, if they are arrested by the thyroidal quality control system, will manifest as a quantitative defect. In the Netherlands the bovine TSH administration test is not used for diagnosis because triggering of autoimmune responses against human TSH is a plausible side effect. Whether the test will be used when recombinant human TSH becomes available is a point of discussion. Often the individual TG response to TSH can be determined by comparing plasma TG levels of untreated (high TSH) patients and thyroid hormone treated (normal TSH) patients. Furthermore, the TSH administration test is not discriminating for a TG synthesis defect.

Functional studies

The past

General insight into the function of a protein can come from pathological descriptions of a phenotype where a mutation in the causal related gene is identified. Sometimes the location and type of the mutation within the gene related to the phenotype can be informative on the structure-function relationship. The phenotypic presentation of CH patients with a mutation in their TG gene is diverse with respect to the thyroid hormone status. This diversity occurs even between patients with the same TG mutation illustrating that besides TG more factors are influencing thyroid
hormonogenesis. The animal models for TG synthesis defects offer an alternative way for *in vivo* studies. The functional defect of Dutch goats and Afrikaander cattle are linked to the fact that both models express a severely truncated TG polypeptide still containing important tyrosine residues enabling thyroid hormonogenesis, although less efficiently. In *cog/cog* mice, a mutation in the domain homologous to acetylhydrogenase results in accumulation of TG in the endoplasmic reticulum. *In vivo* studies on structure-function relationship are hindered by the strong quality control on TG protein synthesis within thyroid follicles. The result, an arrest in maturation of TG within the thyrocyte, is seen in almost all identified TG mutations and makes relation of specific regions of TG gene and protein structure to function difficult. Therefore, additional information on specific functional aspects is gathered by studies on *in vitro* models. These are based on isolation of TG protein from thyroid tissue or obtaining TG protein fragments from an artificial expression system. Subsequently the protein is tested in iodination and iodotyrosine coupling assays. This way, features of the hormonogenic sites were studied and TG structure was reported to be of more influence on selectivity of the tyrosine residues for iodination than TPO while the catalyzation by a peroxidase is important for the efficiency of diiodotyrosine formation (30). However, the iodination assays as generally described use high iodine concentrations and as a consequence too many tyrosine residues are iodinated. Therefore these assays are not suitable to study the influence of TG structure on, for instance, selectivity of tyrosine residues for iodination.

The present: this thesis

To study the structure-function relationship of TG in the iodination reaction, a system was needed to attain suboptimal iodination, reflecting more the *in vivo* iodination conditions. A monolayer of human thyroid immortalized cells, HTori-3, was initially selected to serve as both an expression system and a substrate for iodination studies (Chapter 6). The expression of TG fragments was first tested. Roughly, the TG cDNA was divided into 4 overlapping fragments that were transiently transfected into the cells. No TG could be detected in the medium and the cells stained immunohistochemically with anti-TG demonstrating the presence of cytosolic TG. Although the presence of the signal peptide ensured routing to the endoplasmic reticulum, the strong promoter of the expression construct probably caused over-expression and therefore accumulation in the cisternae. This was also confirmed by carbohydrate structures present on the expressed TG molecules. Because of the improper maturation of TG molecules, this system was not further explored. Another expression system, rabbit reticulocyte lysate, was chosen for synthesis of an
N-terminal TG fragment comparable to the truncated TG proteins described in some TG synthesis defects (Chapter 7). The addition of microsomal membranes ensures post-translational modifications such as mammalian N-glycosylation. Furthermore, the resulting glycosylated TG polypeptides are iodine free but can be iodinated in a subsequent assay by adding lactoperoxidase, a $\text{H}_2\text{O}_2$ generating system and a low amount of RAI, guaranteeing suboptimal iodination conditions. The results show that, comparable to the \textit{in vivo} situation, attachment of carbohydrate structures to the N-terminal TG polypeptide is necessary for effective iodination.

The future

The expression of glycosylated TG fragments and subsequent iodination as described in Chapter 7 needs further examination. Apart from studies on efficiency of iodination, ability to couple iodotyrosyl residues can be studied. Moreover, by using differently labeled TG fragments in the iodination assay interchain coupling events can be studied. These studies can be done for wildtype TG as well as for mutated TG molecules. Furthermore, TG fragments which are expressed, glycosylated and iodinated under controlled conditions can be used in other functional studies as on the transcription regulating function reported for TG (Chapter 1).

\textit{General conclusion}

This thesis presents the complete update of the human TG mRNA coding sequence and the resulting amino acid sequence and provides a useful tool for mutation analysis and studies on the structure-function relationship of TG. The extended knowledge on the heterogeneity of the TG transcript population and the TG polypeptide structure resulting from the various polymorphisms, additional alternative splice transcripts and differential use of polyadenylation cleavage sites, gives an extra dimension to the research on the structure-function relationship of TG. Additionally, the processes involved in TG maturation and its strong quality control are discussed. In a heterogeneous patient group with a putative TG synthesis defect, mutation analysis failed to elucidate a mutation in the TG cDNA. Because of the variability in clinical presentation in this group of patients we hypothesize that defects in other proteins, acting in the TG synthesis and maturation processes, can explain the molecular background of dyshormonogenesis within this group. In the search for yet unknown thyroid specific proteins and proteins that are not yet related to thyroid function, Serial Analysis of Gene Expression serves as a promising technique and probably will reveal candidates for mutation analysis in the group of hypothyroid patients with a putative TG synthesis defect.
REFERENCES


