New insights in the human thyroglobulin structure
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Citation for published version (APA):
GENERAL INTRODUCTION
The thyroid gland & thyroid hormones

Historical aspects

Descriptions of a 'swelling in the neck', that nowadays can be linked to a goitrous thyroid gland, has been known for millennia (1). The first anatomical sketches of the thyroid gland located in neck area are from Leonardo da Vinci, dated around 1500. Magnus-Levi (1895) described the effects of eating thyroid tissue on the oxygen consumption in man and Gudematsch (1912) reported that feeding of thyroid tissue to amphibians hastens metamorphosis while removal of the thyroid from larval forms prevents it (2,3). Later, after much speculation, it was generally accepted that the thyroid gland exerts an influence on metabolism and on development (4). Indication that hormones were responsible for these influences came from the elucidation of hormone structures isolated from thyroid tissue: thyroxine ($T_4$) and 3,5,3'-triiodothyronine ($T_3$). It took another 20 years before it was proven that $T_3$ is the biologically active thyroid hormone (5).

The location of the bilobular thyroid gland in man is shown in Figure 1. A close-up shows the functional structures within the thyroid gland, i.e. the follicles, composed of a single layer of secretory polarized epithelium cells (thyrocytes), surrounding a lumen with their apical membranes.

Figure 1 Thyroid gland.
Location and shape of the thyroid gland (black)(image by technetium uptake in hyperthyroid patient). The thyroid gland consists of follicular structures (F) with epithelium cells (thyrocytes TH) bordering a large lumen (L)(thin section through a normal thyroid gland of goat stained with hematoxylin-eosin (x 100)).
**Chapter 1**

**Thyroid hormone action**

Thyroid hormones are synthesized in the thyroid gland and regularly secreted to the circulation in daily amounts of about 100 nmol T$_4$ and about 5 nmol T$_3$. The major part of the biologically active T$_3$ pool in blood plasma is formed in the liver and kidney by outer-ring deiodination of T$_4$ (6,7).

Thyroid hormone synthesis is under control of the hypothalamic-pituitary axis. The hypothalamic thyrotropin-releasing hormone (TRH) stimulates, through interaction with the TRH-receptor, the synthesis and release of anterior pituitary thyroid-stimulating hormone (TSH) or thyrotropin. TSH in turn stimulates, through interaction with the TSH-receptor growth and hormone secretion by the thyroid gland. Negative feedback in this regulation is via inhibition of TRH and TSH secretion by thyroid hormones.

Approximately 99.99% of thyroid hormones circulate in blood plasma bound to transport proteins like thyroxine-binding globulin (TBG), transthyretin and albumin. The bound fraction is in equilibrium with the unbound fraction. The free fraction (fT$_4$), is taken up by cells that intracellularly convert T$_4$ into the biologically active T$_3$.

Thyroid hormonal activity is mediated by specific nuclear T$_3$ receptors in target tissues, ligand-dependent transcription factors belonging to the nuclear hormone receptor superfamily that includes steroid, retinoic acid and vitamin D3 receptors (8,9). At least three isoforms of thyroid hormone receptors (TRs) are able to bind T$_3$, i.e. TR$\alpha$1, TR$\beta$1 and TR$\beta$2. These TRs and other non-binding isoforms like TR$\alpha$2, are the expression products from two genes, $\alpha$ and $\beta$, after differential use of promoters and alternative splicing events. DNA binding occurs via heterodimerization of different TR isoforms or in partnership with either the retinoid X receptor or the retinoic acid receptor. The receptors bind directly to specific DNA recognition sequences, thyroid hormone response element, in the promoter region of target genes in a complex with other proteins such as co-repressors. Ligand binding generally leads to dissociation of the co-repressors, recruitment of co-activators and dissociation of the ligand-receptor complex from the DNA (10). All these events finally result in the alteration of the transcription initiation rate of the thyroid hormone target gene.

Thyroid hormones are important for control of the metabolic rate and oxygen consumption, and in children they have additionally important effects on growth and development. Growth is thyroid hormone-dependent as T$_3$ exerts its influence at different levels in skeletal growth via growth hormone and insulin-like growth factor-1 (IGF-1). Also brain maturation is strongly thyroid hormone-dependent, and interaction between T$_3$ and nerve growth factor has been suggested (11,12). Already
in early stages of human and rat development, T₃, putatively from maternal origin, is linked to fetal brain development (13, 14).

Because hypothyroidism, i.e. shortage of thyroid hormone, is a frequent disease (1:3000), relatively easy to detect and moreover relatively easy to treat, a neonatal screening procedure is operational in most developed countries and in the Netherlands since 1981. Within one week after birth a blood sample is taken by heel puncture and is initially analyzed for T₄ level. This procedure ensures early replacement therapy with thyroxine for hypothyroid newborns and largely prevents related developmental problems of brain.

*Normal thyroid hormone synthesis and the role of thyroglobulin*

In figure 2 (this Chapter and on cover) a schematic thyroid follicle is depicted, composed of cells enclosing a lumen. Some cell organelles and characteristic proteins are drawn in the first cell. In five other cells, the processes important for thyroglobulin synthesis and thyroid hormone synthesis are emphasized (the localization of these processes is indicated in yellow on the cover).

All six numbered cells are described in more detail:

1. **The thyrocyte in general**

   Thyrocytes are polarized epithelium cells that form follicular structures and their basal membranes are exposed to the blood circulation. The apical membranes and the follicular lumen are not in direct contact with the blood circulation as tight junctions [TJ] connect the cells to each other. Each thyrocyte contains cell organelles like endosomes [E], endoplasmic reticulum [ER], a Golgi apparatus [G], lysosomes [L], mitochondria [M], a nucleus [N], peroxisomes [P], bound and free ribosomes [R]. The follicle can be considered an autonomous thyroid hormone production unit and proteins involved in this process are schematically presented. The receptor [TSH-R] for thyrotropin [TSH], a seven transmembrane protein receptor, is located in the basal membrane. It activates the cAMP pathway via trimeric G proteins and, at high concentrations of TSH, the inositolphosphate pathway. Furthermore, the IGF-1 receptor [IGF-1-R] is present in the basal membrane, a tyrosine kinase receptor that is activated by IGF-1 [IGF-1]. The sodium iodide symporter [NIS] is also located in the basal membrane. In the apical membrane, the thyroid peroxidase [TPO], one or more NAD(P)H oxidases [Tox] and pendrin [Pendrin] are present and transcription factors like Pax8 [8], TTF-1 [1] and TTF-2 [2] are functional in the nucleus. The follicular lumen mainly contains thyroglobulin [TG].
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2 The nucleus: TG gene
The TG gene is located on chromosome 8q24.3-8q24.4 (15,16) and is over 300 kb long (17). The genomic organization of 48 exons has recently been elucidated by Targovnik and co-workers (Chapter 2). The gene contains introns of up to 64 kb (17) and the largest one even contains another gene (18). The exon sequences comprise only 2.5% of the gene sequences. The 5' and 3' part of the TG gene seem to differ in evolutionary origin (19). In the first 30 kb the exon/intron ratio is 1/10, comparable with other mammalian genes, whereas in the remaining 270 kb the ratio is 1/47.
There are many factors that control TG gene expression. TSH, insulin and IGF-1 act synergistically to stimulate transcription. Epidermal growth factor, interferon-γ, tumor necrosis factor and retinoic acid are inhibitors of TG transcription. This transcription regulation works via transcription factors like TTF-1, TTF-2 and Pax8 (20) indicated in the figure and via a cAMP-responsive-element-binding (CREB) protein and a CREB associated factor (21). These factors bind to the promoter region of the TG gene on their characteristic consensus sequences.
The human TG gene encodes an 8.7 kb TG messenger RNA (mRNA)(22). This mRNA consists of a 5'UTR of 41 bp, followed by a coding DNA (cDNA) of 8307 bp with a single open reading frame and a 3'UTR ranging from 101 up to 120 bp. The cDNA as deduced from human TG mRNA, was reported in 1987 to contain 8304 bp (23), but has been extensively revised (this thesis).
The pool of TG mRNAs isolated from thyroid tissue is very heterogeneous, due to polymorphisms, alternative transcription processes and variability in the used polyadenylation cleavage site (this thesis).

3 The endoplasmic reticulum: TG translation
Formation of the translation complex, here outlined by the 40S and 60S ribosomal units, starts with recognition of a so-called CAP structure on the 5'end of the 8.7 kb mRNA transcript. When the first 19 amino acids of the primary structure are linked in the translation complex, this amino acid stretch functions as a signal peptide [SP] to direct the preliminary TG polypeptide to the ER. This SP is spliced off, as it is not present on TG molecules isolated from thyroid tissue and on the N-terminal TG protein fragment synthesized in a baculovirus expression system (24).
On the growing TG polypeptide chain N-glycosylation is initiated. A preformed precursor oligosaccharide (composed of 2 N-acetylglucosamine, 9 mannose and 3 glucose residues [on cover fuchsia, lilac and pink, respectively]) is transferred en bloc to asparagine residues positioned in the Asn-X-Ser/Thr consensus (where X can be any amino acid except proline). Before export from the ER, removal of three glucose
and one mannose residues occurs (25).
The N-terminal 2168 amino acids have a highly organized internal structure with
three types of cysteine-rich repeats. The C-terminal 581 amino acids contain
relatively more tyrosine residues. Most of the 123 cysteine residues in the TG
polypeptide are involved in the formation of intrachain disulfide bonds [S-S]. The
oxidizing environment of the ER fosters the disulfide bond formation that stabilizes
the nascent TG protein (26).
The ER quality control prevents premature export of incompletely or improperly
folded proteins from the ER and initiates the removal of misfolded proteins (27,28).
Part of this control (29,30) is the protective association with molecular chaperones
such as calnexin, BiP and GRP94 which also enhances stabilization and assembly into
TG homodimers. Properly synthesized TG is transported in vesicles to the Golgi
apparatus.

Figure 2 Follicular processes involved in thyroglobulin and thyroid hormone synthesis (next two
pages).
A follicle is schematically depicted with a general thyrocyte (cell 1), processes in different
organelles (cell 2: nucleus, cell 3: endoplasmatic reticulum, cell 4: Golgi apparatus, and cell 5:
apical membrane and follicular lumen), and pathways for TG internalization and secretion of
thyroid hormones and TG (cell 6: from follicular lumen to circulation). See text for further
explanation and cover for color image (clockwise).
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4 The Golgi apparatus: further TG modification
Extensive trimming of the precursor oligosaccharides on TG occurs resulting in the so-called core region, consisting of two N-acetylglucosamine and three mannose residues. After efficient glycosylation, sixteen of the twenty putative N-glycosylation sites present in the TG protein monomer bear carbohydrate structures. The addition of galactose, sialic acid and fucose completes the terminal region and defines the different types of carbohydrate structures (31). In human TG, two additional carbohydrate structures have been reported to be attached to serine and threonine residues. Although not completely characterized, one probably contains galactosamine and the other is described as a chondroitin sulphate unit (32,33).
Other post-translational events also take place during TG maturation like sulphation and phosphorylation (not depicted). In human TG, sulphate residues are present on carbohydrate structures (complex and chondroitin sulphate)(34,35) and on tyrosine residues (36). Phosphate residues linked to carbohydrate structures, primarily in the form of mannose-6-phosphate, and to serine and tyrosine residues have been reported (37,38). Also, purified TG is able to autophosphorylate serine residues in in vitro experiments (39).
Newly synthesized TG attaches to an asialoglycoprotein receptor [ASPGR] and is transported to the follicular lumen. At the apical membrane, a membrane bound sialotransferase and TPO are suggested to sialylate and iodinate the TG, allowing its release from the ASPGR into the follicular lumen (40).

5 The apical membrane and follicular lumen: iodination of TG and coupling of iodotyrosine residues
After vesicle transport to the apical membrane, the TG homodimers are secreted into the follicular lumen. Essential players for TG iodination and coupling reactions are:
- NIS in the basal membrane which imports iodide into the thyrocyte linked to the Na⁺ gradient (41)
- Pendrin that presumably functions as an chloride / iodide transporter in the apical membrane (42,43)
- TPO, a heme containing enzyme, that catalyzes both iodination and coupling reactions, localized in the apical membrane (44)
- Two or more thyroid NAD(P)H oxidases at the apical membrane, generating H₂O₂ that is necessary for TPO functioning (45,46,47,48).
The normal human TG monomer contains 66 tyrosine residues of which on average 18 are iodinated (when TG contains 0.5% iodine) to mono- or diiodotyrosines (MIT or DIT). Coupling involves a reaction between two DIT residues or (less efficiently)
between one MIT and one DIT residue, to form T₄- or T₃-residues, respectively. Coupling, either inter- or intrachain, is restricted to specific iodotyrosyl residues that have to be in a defined spatial configuration. Whether this is an exclusive intrachain or an interchain event is not known. Intrachain coupling is presented in figure 2. The efficiency of the coupling reaction depends on the native structure of TG. The tyrosyl residue that contributes the iodophenolic outer ring of T₄ is the 'donor', whereas the 'acceptor' tyrosyl residue contributes the iodophenolic inner ring and is also known as hormonogenic site. After coupling, the acceptor sites carry T₄ and the donor sites are left with dehydroalanine [DHA]. Among species the hormonogenic sites are homologous and in human TG the tyrosine residues on positions 5, 1291, 2554 and 2747 have been described to carry T₄ of which tyrosine 5 [Y5] is the first to be iodinated. The donor sites are less well defined and for human TG the tyrosine residues on positions 130, 847, 1448 have been proposed of which tyrosine 130 [Y130] is often associated with acceptor site 5 (49).

In the follicular lumen a heterogeneous population of TG molecules is present. Heterogeneity exists with respect to glycosylation, sialylation, phosphorylation, sulphation and iodination. If alternatively spliced TG mRNA transcripts that exist in thyroid cells are translated, they further contribute to the heterogeneity of the TG population. TG is synthesized as a 12S molecule (330 kDa), but forms 19S homodimers and even 27S tetramers. The follicular lumen contains about 30% of total TG in the form of highly multimerized insoluble TG cross-linked by covalent intermolecular bridges (50). Because some chaperones such as BiP, Grp94 and Grp78 are secreted with TG into the follicular lumen it has been suggested that they may be involved in this structural modification of TG. Despite the large iodine content, this form of insoluble TG shows very little ability to synthesize thyroid hormone. Reactive oxygen species and a protein disulfide isomerase appear to cooperate in the rescue of hormonogenetic ability of multimerized TG (51).

6 From follicular lumen to cell: TG to the circulation or back to the Golgi apparatus TG with incompletely processed carbohydrate chains occurs in the normal gland and as iodine organification is positively correlated with glycan completion, these molecules will be poorly iodinated (52). A quality control has been assumed in which specific immature TG molecules are internalized and recycled through the trans-Golgi [G] compartments back to the follicular lumen (53). A receptor-binding-domain has been determined involved in binding of immature TG molecules to a membrane receptor, also called the GlcNAc receptor, in acidic conditions (54,55,56). Protein disulfide isomerase [PDI], originally an ER chaperone, is thought to be a candidate
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receptor, because it is demonstrated to be an apical membrane-associated protein capable of binding to this domain (57). TG-T₄ complexes are removed from the follicular lumen by internalization via micropinocytosis and sometimes via pseudopod ingestion. Micropinocytosis can take place both by nonselective fluid phase uptake and receptor mediated endocytosis (58,59). A candidate for receptor mediated endocytosis is megalin [M] that probably recognizes TG via a heparin binding domain in TG (60,61). Internalization of TG-T₄ complexes is followed by fusion of the endosome [E] with a lysosome [L]. The formed phagolysosomes, acidic in nature, contain all the components for efficient liberation of thyroid hormones from TG. TSH-stimulation increases the lysosomal proteolysis of TG (62). TG is first degraded into discrete iodopeptides and it is evident that in further processing to amino acids [aa] several cathepsins and certain thiol proteases play an essential role (63,64). Yet unsolved dehalogenation processes degrade MIT and DIT residues, not used in coupling reactions, providing free I⁻ ions that can be reused. After liberation from the TG backbone, thyroid hormones [T₄, T₃] are released into the blood circulation to perform their action in the target organs.

Apart from thyroid hormone secretion, the normal thyroid gland secretes low amounts of TG into the circulation, as can be detected via immunological assays. In various pathological conditions like primary congenital hypothyroidism (CH), circulating TG concentration may be markedly elevated. Whereas its biological role, if any, in the circulation is unknown, circulating TG is of major diagnostic relevance, both in CH and in the follow up in thyroid cancer care. Three pathways that can be considered as origin for circulating TG are indicated [dotted arrows]. First, newly synthesized TG molecules (devoid of hormone residues) can be released at the basolateral membrane of thyrocytes into the extrafollicular fluid, instead of being secreted into the follicular lumen (65). Secondly, iodinated and hormone bearing TG molecules can be internalized by megalin at the apical membrane and undergo a transcellular vesicular transport to be released into the extrafollicular fluid (transcytosis) (66). During this process, the TG molecules can either stay intact or be partially degraded (67). Thirdly, in pathological conditions the iodinated and hormone bearing TG molecules are thought to leak via the tight junctions [TJ] into the extrafollicular fluid, because of morphological alterations of the follicles and of the tight junctions (68). Investigation of the hormonal content of circulating TG in various pathological situations revealed the presence of T₄ and T₃, pointing to the transcytosis and leakage pathways in thyroid disease (69,70).
It has recently been shown that follicular TG may regulate the process of gene transcription. It selectively suppresses expression of TTF-1 [1], TTF-2 [2] and Pax-8 [8], thereby altering expression of the TG, TPO, NIS and TSH-R genes, in that way counter-regulating TSH (71,72,73,74,17,18,19). Recently, an enhancing effect of TG is found on the expression of pendrin (43). The asialoglycoprotein receptor [ASGPR] is involved in this transcriptional signaling aside from its role in prevention of preliminary endocytosis (40).

Impaired thyroid hormone synthesis

Neonatal screening on congenital hypothyroidism
In newborns hypothyroidism, if untreated, will lead to irreversible mental retardation and stunted growth. There is a relation between the onset of T₄ - suppletion and mental development: when children with CH are treated earlier they show less difference in IQ compared to age matched normal controls (75,76). Low neonatal T₄ concentration, particularly when caused by thyroid agenesis, increases the severity of cognitive and motor deficits (77).

National screening on hypothyroidism in newborns is operational in The Netherlands since 1981. Within the first week after birth a blood sample is taken by heel puncture and the T₄ concentration is determined. When T₄ is extremely low, the newborn is immediately referred to a paediatrician. When T₄ is low, subsequently TSH and TBG concentration (latter since 1993) are determined from the same bloodspot and if indicated, the child is also referred. Most neonates affected with hypothyroidism lack specific clinical features, but this screening procedure ensures early treatment and largely prevents developmental problems caused by hypothyroidism.

Etiology of congenital hypothyroidism
Congenital hypothyroidism can be classified as transient or permanent. Transient CH spontaneously and completely disappears in several weeks to months after birth.

Permanent CH requires lifelong thyroid hormone treatment. The origin of the defect causing permanent CH can vary: primary CH concerns all defects originated in the thyroid, while secondary / tertiary CH concerns disturbances in the hypothalamic-pituitary regulatory axis of the thyroid. A high blood plasma TSH concentration is indicative for primary CH, whereas TSH is low to normal in the case of secondary / tertiary CH and diagnosis can only be made by demonstrating a low fT₄ concentration.

In the first Dutch screening cohort from 1981-1982, 97% of all cases of permanent CH (78) have been characterized. In 12% of the cases a low plasma TSH
concentration indicated a secondary / tertiary CH. Primary CH was diagnosed in 85% of the cases with permanent low T₄. The majority of primary CH cases, 71%, was linked to a maldevelopment of the thyroid gland, i.e. a- or dysgenesis. In 14% of the cases, dyshormonogenesis of the thyroid gland was the cause of CH.

**Thyroid dyshormonogenesis**

Theoretically, the origin of thyroid dyshormonogenesis (TD) can be at all steps in the metabolic pathways of the follicular cells especially in the proteins directly involved in thyroid hormonogenesis (Fig 2) (79,80,81). Because of early treatment nowadays, goiter of secondary appearance sporadically develops and thyroidectomy has become rare. As a consequence thyroid tissue is no longer available for biochemical or histological diagnosis, and blood is the only source for biochemical diagnosis. However, to be able to screen for defects at the molecular genetic level in any of the proteins involved in thyroid hormonogenesis, the candidate genes have to be known.

From 1987 up till now, the coding DNA sequence (cDNA) from only six of these proteins is elucidated (Table 1). These proteins are known to be involved in activation of the thyrocyte, in iodide transport and in iodination and coupling reactions. No specific proteins involved in the dehalogenation process (iodine recycling) are identified yet. The number of kindreds with molecular defects causally linked to TD is presented per protein (Table 1). A lot of kindreds have been successfully screened for mutations in the TPO gene subsequent to the elucidation of the TPO cDNA in 1987. Although the TG cDNA was also elucidated in 1987, only five mutations have been determined in six different kindreds. Undoubtedly, the exceptional size both of the TG gene that consists of 48 exons and of the cDNA contribute to the fact that not many patients with a putative TG synthesis defect have been checked for mutations in the TG gene. In six cases clinicopathologically diagnosed as having a TG synthesis defect we analyzed the TG cDNA and no mutations were present in the TG cDNA (this thesis). This is probably due to the fact that the defect is located in other proteins, involved in the synthesis of a functional TG protein.
Dyshormonogenesis due to a mutation in the TSH receptor has a variable clinical picture. Phenotypically, affected patients present TSH hyporesponsiveness. They present with normal or hypoplastic thyroid glands associated with high plasma TSH concentrations, normal or low plasma $T_4$ and $T_3$ concentrations, and normal plasma TG levels (85). Genetically, patients are described as compound heterozygous for loss-of-function mutations or premature termination of the protein.

CH patients with a mutation in the NIS gene phenotypically present an iodide uptake defect. They present high plasma TSH concentrations and low to normal $T_4$ concentrations. When a radioactive iodine (RAI) image is made, a low amount of RAI is accumulated in the thyroid gland. As the NIS protein is also expressed in the saliva glands, uptake of RAI occurs when NIS is properly functioning. This results in a RAI saliva-to-blood ratio of >2.9 (78). A RAI saliva-to-blood ratio reaching unity is an extra parameter implicating a total iodide uptake defect. Genetically, patients have been described as either homozygous or compound heterozygous for mutations resulting in an amino acid change or premature termination of the protein.

A phenotypic total or partial iodide organification defect can also be the cause for dyshormonogenesis. Patients present high plasma TSH concentrations, low plasma $T_4$ concentrations and very high plasma TG levels. Characteristic is a more than 20% discharge of the RAI accumulated in the thyroid after administration of perchlorate, which blocks the iodide uptake by the thyroid gland. Depending on the percentage of discharge, the distinction between a total and a partial iodide organification defect is
made. Theoretically, an iodide organification defect can result from a defect in the thyroid peroxidase or in the thyroid oxidase. The enzyme thyroid peroxidase, TPO, is a heme binding transmembrane protein located in the apical membrane that catalyzes the iodination and coupling reactions. In clinical practice, mutations in the TPO gene are linked to total iodide organification deficiencies and vary from nucleotide substitutions to mutations causing a frameshift. All defects inherited in an autosomal recessive way. Recently, several thyroid specific oxidases have been identified. Their definite role in thyroid (dys)hormonogenesis can be established when loss-of-function mutations in patients with a partial or total iodide organification defect are identified.

A mutation in the PDS gene is phenotypically linked to Pendred’s syndrome. This syndrome is clinically described as an impaired hearing defect since infancy, often caused by a Mondini type malformation of the cochlea or enlarged vestibular aqueduct (86,89). In a subpopulation a moderately enlarged thyroid gland, euthyroidism, and a partial discharge of administered RAI after administration of perchlorate are additional thyroid specific characteristics. The transmembrane protein encoded by the PDS gene, pendrin, presumably functions as a transporter for iodide and chloride (42) and is located in the apical membrane (43). Its role in the pathogenesis of Pendred’s syndrome remains to be elucidated, as well as its role in the embryogenesis and function of the cochlea. Since its cDNA identification in 1997, a lot of mutations including two frequent missense mutations have been described in the PDS gene. Also patients clinically defined with Pendred’s syndrome have been reported with no obvious mutations in the PDS gene.

A minority of the cases of thyroid dyshormonogenesis is caused by mutations in the TG gene, phenotypically known as a TG synthesis defect. The affected patients present a clinicopathological variable picture. They are moderately to severely hypothyroid and usually have low plasma TG levels with respect to the elevated TSH levels. The RAI image shows a high and rapid uptake exceeding the normal 10-30% of the administered RAI present in the gland after 24 hours, and usually no discharge of iodide is noticed upon perchlorate administration. Sometimes, the urine is analyzed for its iodine content and presence of low molecular weight iodinated material (LOMWIOM) is indicative for iodination of polypeptides other than TG (91). In plasma the protein bound iodine (PBI) content can be analyzed and this can also be also indicative for iodination of polypeptides other than TG and mainly contains iodinated plasma albumin. At the molecular level five mutations have been described, varying from nonsense and missense mutations to deletions.
**TG synthesis defect**

**Possible defects**

Looking at the process of thyroid hormonogenesis and the prominent role of TG (Fig 2) it seems inevitable that one of the etiologies of CH is a TG disorder. There can be a complete lack of TG in the follicular lumen or TG is present but either dysfunctions in the synthesis of thyroid hormones or fails to be properly internalized and degraded. Theoretically, TG deficiency in thyroid tissue can be caused by a defect at the transcription or the translation level. TG dysfunction can be caused by defective post-translational processes or by defects in the endocytosis-proteolysis pathway. In other words, a TG synthesis defect can be caused by a factor upstream of TG transcription, a defect in a TG transcript and protein or a defect in a protein interacting with TG.

**Clinical description**

A defect in TG synthesis is roughly implicated by the following clinical features: a normally sized or enlarged thyroid gland, low to normal plasma T₄ levels, high plasma TSH levels associated with low or absent TG in plasma, a high and rapid RAI uptake and negative perchlorate test, the presence of iodinated polypeptides other than TG, mainly iodinated plasma albumin, in plasma (PBI) or in urine (LOMWIOM). These features exclude possible defects at the level of the TSH receptor, the sodium-iodide symporter, pendrin, the thyroid peroxidase or the thyroid oxidase (see preceding paragraph).

A clinical test has been described, based on the idea that the TG concentration measured in plasma reflects the TG concentration in the follicular lumen (92). The patient intravenously receives bovine TSH and at different time points TG plasma levels are measured in an immunological assay. When TG plasma levels do not respond to the TSH stimulus (normal subjects have a 3 – 5 fold increase) absence of TG in the follicular lumen is concluded. In some unresponsive patients, mutations in the TG gene are reported and in few of these, accumulation of immunogenic TG is noticed inside the cell. This bovine TSH administration test is not performed in our paediatric clinic because triggering of autoimmune responses against human TSH is a plausible side effect. The use of recombinant human TSH in the diagnostics of a TG synthesis defect has not been reported yet. An overview of all cases reported since 1959 that were diagnosed to have a TG synthesis defect based on the clinical description is available (93).

**Identified defects**

In man, five mutations in the TG gene have been reported. All six patients are homozygous for the mutations and fit the clinical description of a TG synthesis
defect, although the locations of the mutation and the corresponding defects on
protein level are strikingly dissimilar.
A deletion of 68 amino acids from the N-terminal part of the TG protein and a
deletion of 46 amino acids of the middle of the protein, due to the inframe deletion of
exon 4 and exon 30 respectively, have been reported (94,95). Histochemical analysis
of thyroid tissue from the patient with the homozygous exon 30 deletion showed
arrest of TG in the thyrocytes, suggesting that the thyroidal quality control process
largely prevents these TG molecules from entering the lumen (96).
A mutated transcript, T3790C, that on the protein level results in a cysteine to
arginine mutation, was reported in two unrelated patients (97). In one of the patients,
after RAI administration, a high and rapid uptake was measured with an increased
discharge of 37% upon perchlorate administration (98), a phenomenon also seen in
partial iodide organification defects. Biochemical analysis on TG isolated from the
thyroid tissue showed that the TG molecules were presumably retained in the
endoplasmic reticulum.
Two cases have been described in which expression of the mutated transcript results
in a truncated TG molecule. In one case exon 22 contains a premature stopcodon
that after translation results in a truncated protein (99). Thyroid tissue also contains
an alternative splice variant from which exon 22 was deleted. Although this variant of
alternative splicing also has been described in thyroid tissue of normal subjects (100),
it was increased in this patient and after translation it generated TG molecules
containing an internal deletion besides truncated molecules. Histological analysis of
thyroid tissue showed that the majority of TG was present in the thyrocyte. We
reported a patient with a premature stopcodon in exon 7 resulting in an even shorter
TG molecule after translation (Chapter 5). Because $T_4$ levels are detected in the
plasma, it was concluded that the truncated molecule was capable of thyroid
hormone synthesis. Plasma TG levels were low and did not respond to bovine TSH.
Histological analysis revealed that TG was only present within the cell.
In animals, three mutations have been reported. One, in Afrikander cattle, is similar to
one of the human mutations (101). The mutation results after translation in a
premature truncated TG molecule but also TG molecules with an internal deletion are
detected resulting from an upregulated alternative splice event. The TG molecules
were present in the follicular lumen.
In Dutch goats a mutation has been reported that results in the production of an even
smaller N-terminal protein fragment (102). The goats shows a high and rapid uptake
of RAI and no discharge upon perchlorate administration. Iodinated proteins other
then TG were present indicated by LOMWIO in the urine. These goats are euthyroid under high dietary iodine provision although goiter remains. This indicates that the N-terminal part of TG is able to synthesise thyroid hormone.

In cog/cog mice a mutation in the TG gene has been identified (103). It concerns a mutation in the 3' part of the gene and the functional link is not yet elucidated. In this case the TG molecules are present in the ER, probably tackled by the quality control. The human patients are of the pre-screening era or from countries where the neonatal screening was not yet introduced. Man and animals developed goiter and mutational analysis was performed on RNA isolated from surgically removed thyroid tissue.

While TSH was higher than normal in all cases, plasma TG levels were low or undetectable. The T₄ levels varied from hypothyroid to euthyroid values. The goitrous thyroid tissue, sometimes already noticed at birth, probably provided sufficient thyroid hormone in a number of cases, although less efficiently. The variety in thyroid hormone status of patients with a TG synthesis defect as seen between families probably reflects the iodine intake (104).

**Scope of this thesis**

In order to determine the molecular defect in patients presenting permanent primary congenital hypothyroidism supposedly due to a thyroglobulin synthesis defect, we have established the correct 8307 bp coding DNA sequence of human TG including several polymorphisms (Chapter 2). A variety of TG transcripts derives from alternative splice events (Chapter 2) and the variable use of polyadenylation cleavage sites in the 3'UTR (Chapter 3).

The TG cDNA of 7 patients was screened for mutations. In six of them no link was found between the TG cDNA and the disease (Chapter 4) whereas in one patient a nonsense mutation was found causing a truncation of the TG molecule (Chapter 5). Searching for an in vitro model for structure-function studies of human thyroglobulin, TG fragments were expressed in a monolayer of a human thyroid cell line (Chapter 6) and in a cell free coupled transcription / translation system (Chapter 7). In these chapters, glycosylation of the expressed TG fragments was used either as a tool to determine the post-translational pathways in the cells or as a prerequisite for functional iodination studies.
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Chapter 1


