A SINGLE CHONDROITIN 6-SULFATE OLIGOSACCHARIDE UNIT AT SER-2730 OF HUMAN THYROGLOBULIN ENHANCES HORMONE FORMATION AND LIMITS PROTEOLYTIC ACCESSIBILITY AT THE CARBOXYL TERMINUS

POTENTIAL INSIGHTS INTO THYROID HOMEOSTASIS AND AUTOIMMUNITY*

Marisa Conte, Alessia Arcaro, Daniela D’Angelo, Arielle Gnata, Gianfranco Mamone, Pasquale Ferranti, Silvestro Formisano, and Fabrizio Gentile

From the Dipartimento di Scienze per la Salute, Università del Molise, Via F. De Sanctis, Campobasso 86100, the Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università di Napoli Federico II, Via S. Pansini 5, Napoli 80131, Istituto di Scienze dell’Alimentazione del Consiglio Nazionale delle Ricerche, Via Roma 52a/c, Avellino 83100, Italy, and the Dipartimento di Scienza degli Alimenti, Università di Napoli Federico II, Parco Gussone, Portici 80055, Italy

Running Title: Chondroitin 6-sulfate chain of human thyroglobulin

† To whom correspondence should be addressed: Tel. +39-0874-404852; Fax: +39-0874-404778; E-mail: gentilefabrizio@unimol.it

We localized the site of type D (chondroitin 6-sulfate) oligosaccharide unit addition to human thyroglobulin (hTg). hTg was chromatographically separated into chondroitin 6-sulfate-containing (hTg-CS) and chondroitin 6-sulfate-devoid (hTg-CS\(d\)) molecules, on the base of their D-glucuronic acid content. In an ample number of hTg preparations, the fraction of hTg-CS in total hTg ranged from 32.0 to 71.6%. By exploiting the electrophoretic mobility shift and metachromasia conferred by chondroitin 6-sulfate upon the products of limited proteolysis of hTg, chondroitin 6-sulfate was first restricted to a carboxyl-terminal region, starting at residue 2514. A single chondroitin 6-sulfate-containing nonapeptide was isolated in pure form from the products of digestion of hTg with endoproteinase Glu-C, and its sequence was determined as LTAGXGLRE (residues 2726-2734, X being Ser2730 linked to the oligosaccharide chain). In an in vitro assay of enzymatic iodination, hTg-CS produced higher yields of 3,5,5′-triiodothyronine (T3) (171%) and 3,5,3′,5′-tetraiodothyronine (T4) (134%), than hTg-\(CS_d\). Unfractionated hTg behaved as hTg-CS. Thus, chondroitin 6-sulfate addition to a subset of hTg molecules enhanced the overall level of T4 and, particularly, T3 formation. Furthermore, the chondroitin 6-sulfate oligosaccharide unit of hTg-CS protected peptide bond Lys2714-Gly2715 from proteolysis, during the limited digestion of hTg-CS with trypsin. These findings provide insights into the molecular mechanism of regulation of the hormonogenic efficiency and of the T4/T3 ratio in hTg. The potential implications in the ability of hTg to function as an autoantigen, and into the pathogenesis of thyroidal and extra-thyroidal manifestations of autoimmune thyroid disease are discussed.

Thyroglobulin (Tg)\(^{1}\), the molecular site of thyroid hormone formation, is a large homodimeric glycoprotein with a \(M_r\) of 660,000. It is also a major antigen, involved in the pathogenesis of thyroid autoimmunity. In fact, experimental autoimmune thyroiditis (EAT) can be induced in genetically susceptible mice, by immunization with human thyroglobulin (hTg) in complete adjuvant, and autoantibodies to hTg are found in the blood of humans affected with autoimmune thyroid disease (AITD), even though their pathogenic significance is unclear (1). Several post-translational modifications contribute to the molecular microheterogeneity of hTg, including iodination, glycosylation, phosphorylation and sulfation (reviewed in Ref. 2). Iodine addition and hormone formation at specific sites have the most obvious implications for thyroid function, but the effects of glycosylation on the hormone-forming
efficiency at specific sites and on the antigenicity of Tg have also been documented (3, 4). hTg is modified with the addition of several oligosaccharide units of different kinds, among which N-linked type A (high-mannose) and type B (complex) units have been characterized best, as to their composition and localization (5-7). Type C units are linked to serine and threonine by O-glycosidic bonds and contain D-galactosamine (5). Another O-linked, type D oligosaccharide unit was described, which was composed of a repeating D-glucuronic acid-N-acetyl-D-galactosamine sulfate disaccharide, attached to the polypeptide chain through a D-galactosyl-D-xylosyl-serine linkage region (8). Further study indicated that the repeating disaccharides were of the chondroitin 6-sulfate type (9, 10). However, the number and localization of type D oligosaccharide units in hTg were not determined.

A number of studies have documented the influence of oligosaccharide chains in the processing and/or presentation of glycoproteic antigens by antigen-presenting cells (APCs) (11-16), and the involvement of chondroitin 6-sulfate oligosaccharide chains in the modulation of cellular immune responses (17-20). Prompted by these observations, we began to determine in detail the number and localization of chondroitin 6-sulfate oligosaccharide unit(s) in hTg, as a basis for further studies on their influence on the immunopathogenicity of hTg in a murine model of EAT. We found that the addition of chondroitin 6-sulfate unit(s) is a major source of molecular microheterogeneity of hTg. In dozens of hTg preparations examined, it was regularly found in a percentage of hTg molecules varying from 32.0% to 71.6% of total. Moreover, we determined that a single chondroitin 6-sulfate unit per hTg polypeptide chain is linked to Ser2730. We also observed changes in the hormone-forming efficiency of hTg, and in the proteolytic accessibility of the extreme carboxyl-terminal region of hTg, associated with the presence of the chondroitin 6-sulfate oligosaccharide chain. The former observation contributes to delineate a general mechanism, by which modifications in the composition and number of N-linked and O-linked oligosaccharide units determine changes in the hormone-forming efficiency of the main hormonogenic domains at both hTg extremities, while the latter finding supports the potential role of the chondroitin 6-sulfate oligosaccharide unit in modifying the susceptibility of hTg to processing by APCs and the repertoire of hTg epitopes thus generated.

**EXPERIMENTAL PROCEDURES**

**Materials** - Thermolysin from *Bacillus thermoproteolyticus rokko* (EC 3.4.24.4), L-1-tosylamide-2-phenylethylchloromethyl-treated trypsin from bovine pancreas (EC 3.4.21.4), endoproteinase Glu-C from *Staphylococcus aureus* (EC 3.4.21.19), lactoperoxidase (LPO) from bovine milk (EC 1.11.1.7), and glucose oxidase from *Aspergillus niger* (EC 1.1.3.4) were purchased from Sigma. Protease-free chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4) was from Roche Applied Science. Aminopeptidase M from porcine kidney (EC 3.4.11.2) and Pronase from *Streptomyces griseus* were from Calbiochem. Sephaeryl S-300 HR, HiTrap™ Q Sepharose HP, DEAE-Sepharose Fast Flow, and Sephadex G-50 fine were obtained from GE Healthcare. Bio-Gel P-2 and electrophoresis products were from Bio-Rad. Immobilon P membranes and Centrigrad 30 concentrators were from Millipore (Vimodrone, Italy). HPLC-grade solvents were from Carlo Erba (Milan, Italy). BCA protein assay reagent was from Pierce. 3,3′,4′-diethyl-9-methyl-4,5,4′,5′-dibenzo[b,d]thiacyanocarbocyanine (DMTCC, Stains All™) was from ICN Biomedicals (Milan, Italy). Solid-phase radioimmunooassay kits for total 3,5,5′-triiodothyronine (T3) and 3,5,3′,5′-tetraiodothyronine (thyroxine, T4) were from Diagnostic Products Corp. (Los Angeles, CA). Marker kit MW-SDS-17S and other analytical grade chemicals were from Sigma.

**Purification of hTg** - hTg was prepared as described (21), from informed euthyroid patients hemilaryngectomized for non-thyroidal disease, and patients undergoing thyroidectomy for nonfamilial, simple, or multinodular goiter. Protein concentration was assayed by measuring the absorbance at 280 nm, using an extinction coefficient of 10 cm⁻¹ for 1% solution. Iodine content was assayed as described, using L-thyroxine as the standard (22).

**Ion-exchange Chromatography of hTg on HiTrap™ Q-Sepharose HP (Q-IEC)** – hTg molecules containing type D (chondroitin 6-sulfate) oligosaccharide units (hTg-CS) were separated from residual hTg molecules, devoid of type D units (hTg-CS0), by ion-exchange chromatography on trimethylamino-substituted Q-Sepharose (Q-IEC) using 5-ml HiTrap™ Q-Sepharose HP columns, equilibrated in 0.025 M Tris/HCl, pH 7.4 (buffer A). Up to 20 mg of hTg in buffer A, plus 0.05 M NaCl, were
applied to a column. After washing with buffer A, a linear gradient from 0 to 100% of buffer B (1.2 M NaCl in buffer A) was developed in 24 min at the flow rate of 2.5 ml/min. One-ml fractions were analyzed or stored at -80 °C until use.

**Comparative Analysis of Q-IEC Fractions of hTG – D-Glucuronic acid was assayed in Q-IEC fractions by the meta-hydroxybiphenyl method (23), using the same compound (0.5 µm) as the standard. Duplicate samples of 30-500 µg of hTG were dialyzed against 0.01 M NH$_4$HCO$_3$, dried in centrifugal evaporator, and redissolved in 0.2 ml of double-distilled H$_2$O in borosilicate Pyrex tubes. Samples were processed by the addition of 1.2 ml of 0.0125 M Na$_2$B$_4$O$_4$•10 H$_2$O in H$_2$SO$_4$ and, after heating in boiling water bath for 5 min and cooling on ice, 0.02 ml of 0.15% meta-hydroxybiphenyl in 0.5% NaOH, after which the optical absorbance at 520 nm was read. Correction for aspecific color development was provided by a replicate set of samples, which were treated identically, with the exclusion of meta-hydroxybiphenyl in the 0.5% NaOH reagent. Total neutral hexoses were assayed in duplicate samples of 20-300 µg of hTG by the anthrone method (24), using D-galactose (0-20 µg) as the standard. Sialic acid was assayed in duplicate samples of 40-500 µg of hTG by the thiobarbituric acid method (25), using N-acetyleneuraminic acid (0-15 µg) as the standard. Iodine was assayed in duplicate samples of 30-200 µg of hTG, using the method cited (22).

**Enzymatic In Vitro Iodination of hTG and Analysis of the Iodine, T3 and T4 Content –** hTG from goiters, with an iodine content not exceeding 0.09% on a weight basis, was iodinated enzymatically in vitro. Bulk iodination of 20 mg of unfractonated hTG, at the concentration of 0.45 g/liter, was performed in 0.02 M imidazole/HCl, pH 7.0, using 2 µg/ml of LPO from bovine milk, 4 × 10$^{-5}$ M potassium iodide, 1 × 10$^{-3}$ M D-glucose, and 0.19 µg/ml of glucose oxidase from A. niger. Iodination was stopped with 0.05 M 2-mercapto-1-methylimidazole (MMI). Comparative iodination of unfractonated hTG and of hTG-CS$_0$ and hTG-CS$_-$ subfractions of the same hTG preparation was performed with 0.65 mg of each protein, under identical conditions, except that 7.5 × 10$^{-5}$ M potassium iodide, and 0.21 µg/ml of glucose oxidase were used. Aliquots of 0.1 mg of hTG were withdrawn at 5, 15, 30, 50, 70 and 90 min, supplemented with MMI, dialyzed against 0.01 M NH$_4$HCO$_3$, 5 × 10$^{-3}$ M NaCl, and assayed in duplicate for protein content, using the BCA protein assay reagent and bovine serum albumin as the standard, and for iodine content, as already described. For the assay of T4 and T3, 10-15 µg of hTG were hydrolyzed at 37 °C with Pronase, at the enzyme/substrate weight ratio of 1:1, in 0.2 ml of 0.1 M Tris/HCl, 0.05 M MMI, pH 8.0, to which 15 µl of toluene were added. After 24 h, aminopeptidase M was added, at the enzyme/substrate weight ratio of 1:10, and digestion was prolonged for 24 h at 37 °C, after which T4 and T3 were measured by solid-phase radioimmunoassays in antibody-coated tubes (26). In both assays, $^{125}$I-labeled T4 or T3 competed with the respective hormone in the test samples for antibody sites. After the tubes were decanted and radioactivity was measured, the T4 or T3 concentration was obtained by interpolation from a calibration curve.

**Limited Proteolysis of hTG – hTG, at the concentration of 1 g/liter in 0.05 M Tris/HCl, 0.1 M NaCl, pH 7.4, was digested with thermolysin, at the enzyme/substrate weight ratio of 1:50, at 30 °C for 80 min, or with L-1-tosylamide-2-phenylethylchloromethyl-treated trypsin (henceforth referred to as trypsin), at the enzyme/substrate weight ratio of 1:100, at 30 °C for 20 or 40 min. Proteolytic digestion was stopped with 3 × 10$^{-5}$ M antipain, 2 × 10$^{-6}$ M aprotinin, 5 × 10$^{-4}$ M benzamidine, 4 × 10$^{-5}$ M leupeptin, 1 × 10$^{-4}$ M Na-p-tosyl-L-lysine chloromethyl ketone, 2 × 10$^{-5}$ M phenylmethyl-sulfonyl fluoride, 5 × 10$^{-3}$ M EDTA and, in the case of trypsin, soybean trypsin inhibitor, at the inhibitor/enzyme weight ratio of 3:1. Thereafter, concentrated SDS-PAGE sample buffer was added to a final concentration of 0.01 M Tris/HCl, pH 6.8, 1% SDS, 5% 2-mercaptoethanol (v/v), 1.36 M glycerol, 0.0025% bromophenol blue, and the samples were heated in a boiling water bath for 1.5 min and immediately subjected to SDS-PAGE.

**Enzymatic Digestion of Chondroitin 6-Sulfate Oligosaccharide Units of hTG, hTG Subfractions and hTG Proteolytic Fragments with Chondroitinase ABC –** hTG, or its proteolytic fragments in 0.1 M NaCl, 0.05 M Tris/HCl, pH 7.4, were supplemented with equal volumes of 0.1 M sodium acetate, 0.1 M Tris/HCl, pH 8.0. Pooled fractions from the Q-IEC of hTG were dialyzed against the same buffer. All samples were supplemented with 200 milliunits/ml of chondroitinase ABC from P. vulgaris, which degrades chondroitin 4-sulfate and 6-sulfate oligosaccharide chains into...
sulfated disaccharides by hydrolyzing the β-(1→4)-glycosidic bonds between N-acetyl-D-galactosamine 4- and 6-sulfate and D-glucuronic acid in the repeating disaccharide units. The protease inhibitors already indicated with regard to limited proteolysis were added, when not already present, and the samples were incubated at 37 °C for 4 h. Samples to be analyzed by SDS-PAGE were immediately precipitated in methanol/chloroform/water (27), although those to be used for enzymatic iodination in vitro or limited tryptic digestion, following chondroitinase ABC digestion, were subjected to Q rechromatography, as already described, concentrated in Centriprep 30 concentrators and dialyzed against the appropriate buffer.

**Isolation and Sequencing of Glycopeptide hTg-CSgp** – Forty seven mg of hTg-CS were denatured and reduced in 15 ml of 0.3 M Tris/HCl, pH 8.0, 6.0 M guanidine/HCl, 1 × 10⁻³ M EDTA, 0.01 M dithiothreitol at 37 °C for 2 h. The reduced protein was carboxymethylated with a 5-fold molar excess of iodoacetamide, with respect to total –SH groups, at room temperature for 30 min in the dark. Alkylation was stopped with excess dithiothreitol. The sample was dialyzed against 0.05 M sodium phosphate, pH 7.8, and digested with endoproteinase Glu-C (protease V8) from *S. aureus* at the enzyme/substrate weight ratio of 1:100 at 37 °C for 18 h. The sample was adjusted with concentrated solutions to 0.025 M TrisHCl, 0.1 M NaCl, 2.0 M urea, pH 7.4 (buffer C), and loaded onto a 5-ml HiTrap™ Q-Sepharose HP column equilibrated in the same buffer. After washing with buffer C, a gradient was started from 0 to 100% of buffer D (1.2 M NaCl in buffer C) in 55 min, followed by 100% buffer D for 10 min at the flow rate of 1 ml/min. One-ml fractions were monitored for the optical absorbance at 280 nm and D-glucuronic acid content. A single D-glucuronic acid-containing peak was subjected to size-exclusion chromatography on a 1.5 × 100-cm column of Bio-Gel P-2 in 0.01 M NH₄HCO₃. A D-glucuronic acid-containing peak, eluted in the void volume, was lyophilized and further purified by gel chromatography on a 0.5 × 40-cm column of Sephadex G-50 fine in 0.01 M NH₄HCO₃. One-ml fractions were monitored for peptide content, by measuring the optical absorbance at 220 nm, and D-glucuronic acid content, and a single peptide- and D-glucuronic acid-containing peak was lyophilized. A solubilized aliquot was subjected to NH₂-terminal peptide microsequencing. Purity was checked by PAGE in Tris/Tricine/SDS in a 16.5% total acrylamide, 6% N,N'-methylene-bis-acrylamide gel, containing 6.0 M urea (30). Relative molecular mass standards (marker kit MW-SDS-17S, Sigma) were as follows: myoglobin fragments 1-153 (16,950), 1-131 (14,440), 56-153 (10,600), 56-131 (8,160), 1-55 (6,210), glucagon (3,480), and myoglobin fragment 132-153 (2,510). SDS-PAGE was followed by semi-dry transfer to Immobilon P, as described above, for 25 min. The membrane was stained with DMTCC in 50% formamide and destained in tap water (28).
Purification of Carboxyl-terminal Tryptic Fragments of hTg by HPLC – The fragments obtained from the limited digestion of 20 mg of goiter hTg (0.03% of iodine on a weight basis) with trypsin for 20 min, under the conditions described above, were freed from trypsin immediately after digestion by filtration through a 10-ml column of DEAE-Sepharose Fast Flow in 0.025 M Tris/HCl, pH 7.2. hTg fragments were eluted with 0.15 M NaCl in 0.025 M Tris/HCl, pH 7.2, and desalted by gel filtration on a 1.5 × 100-cm column of Bio-Gel P-2, equilibrated with 0.01 M NH₄HCO₃. One-mg aliquots of the fragments were fractionated by reverse-phase HPLC with a Vydac C-4 column (250 × 10 mm, 5 µm) equilibrated in 0.1% (v/v) trifluoroacetic acid in H₂O (solvent A) and containing 2% of 0.07% trifluoroacetic acid in acetonitrile (solvent B). After 2 min at 35% of solvent B, the fragments were eluted with a two-step linear gradient from 35 to 46% of solvent B over 35 min and from 46 to 55.5% over the following 35 min. The flow rate was 1 ml/min. Fractions were analyzed by SDS-PAGE in 4-17% total acrylamide gradient gels under reducing and nonreducing conditions. Corresponding peaks from repeated runs were pooled for further mass spectrometric analysis.

Characterization of Carboxyl-terminal Tryptic Fragments of hTg by Electrospray Mass Spectrometry – ES-MS of peptide h4bisTR were recorded on a Q-TOF Ultima hybrid mass spectrometer (Waters), equipped with an electrospray ion source and operating in positive ion mode. A nanoflow high pressure pump system model CapLC (Waters) was used to deliver a 1 μl/min flow rate of 10% acetonitrile to the mass spectrometer. The spectra were scanned at the speed of 10 s/scan. The source conditions were the following: capillary voltage, 3000 V; cone voltage, 100 V; extractor, 0 V; RF Lens, 60. Raw data were processed using the MassLynx 3.5 software. Mass calibration was carried out using the multiple charged ions from a separate introduction of horse heart myoglobin (average relative molecular mass of 16950.5). The quantitative analysis was performed by integration of the multiple charged ions of the single species. Molecular masses are reported as average values. The mass signals recorded were associated with the corresponding peptides, on the basis of the expected molecular masses, using the Biolynx software (Waters).

RESULTS
We hypothesized that the complexity and extended span of D-glucuronic acid content of these Q2 peaks might reflect the coexistence of heterodimeric hTg-CS, with a single chondroitin 6-sulfate-bearing subunit, and homodimeric hTg-CS. To prove this, pooled fractions of the Q2 peak from the Q-IEC of hTg Mi. N. were dissociated in 0.04 M Tris/HCl, 2.5 M urea, pH 9.0, at 20 °C overnight, as described previously (31), and subjected again to Q-IEC, using a NaCl gradient from 0 to 1.2 mol/liter in 2.5 M urea, 0.055 M Tris/HCl, pH 7.4. As shown in Fig. 2, panel B, the hTg monomers produced by the dissociation of hTg-CS in urea were separated again into peaks Q1 and Q2, eluting at NaCl concentrations of 0.44 and 0.86 mol/liter, respectively. Once again, D-glucuronic acid was restricted in the Q2 peak, and its concentration showed a linear increase from 3.2 to 14.8 mol/hTg mol across the peak span, as opposed to the increase from 3.6 to 24.0 mol/mol of undissociated hTg-CS (Table 1). The Q2 peak of dissociated hTg-CS was superimposed onto the leading component of the Q2 peak of native hTg-CS, with no secondary shoulder at the trailing edge. The Q2/Q1 ratio in hTg-CS dissociated in urea was 72.7% versus 27.3%. These data indicate that the hTg-CS fraction of hTg Mi. N. included both an earlier eluting subfraction of heterodimers, composed of nonchondroitinated (27.3%) and chondroitinated (27.3%) monomers in the 1:1 ratio, and a later eluting homodimeric subfraction, composed only of chondroitinated subunits, and representing the remaining 45.4% of hTg-CS. Thus, hTg-CS was microheterogeneous, both because of the variable number of repeating disaccharide units per chondroitin 6-sulfate chain, which is typical of glycosaminoglycans (GAGs) (32), and because of the coexistence of heterodimeric and homodimeric hTg-CS molecules in hTg-CS-rich goat hTg preparations. When the pooled fractions of the Q2 peak from the Q-IEC of hTg preparation Ca. were digested with chondroitinase ABC and subjected to Q rechromatography, they eluted in a peak superimposed onto the Q1 peak of the initial Q-IEC of native hTg (Fig. 2, panel C). Fig. 1, panels A and B, also shows the native PAGE of the Q-IEC fractions of hTg preparations O. and Ma., evidencing the anodal shift, going from the Q1 to the Q2 peak, because of the added negative charge of D-glucuronic acid residues and sulfate groups of the chondroitin 6-sulfate unit(s).

hTg-CS Has a Higher Efficiency of T3 and T4 Formation than hTg-CS0 and Enhances the Overall Hormonogenic Efficiency of hTg – We investigated the possible influence of the chondroitin 6-sulfate oligosaccharide unit(s) on the hormonogenic function of hTg-CS, in comparison with chondroitin 6-sulfate-devoid hTg (henceforth referred to as hTg-CS0), and unfractionated hTg. Iodination of Tg at the apical pole of thyroid follicular cells occurs by the action of thyroid peroxidase (TPO) (reviewed in Ref. 2). Lactoperoxidase (LPO) is a common alternative for TPO in reconstituted systems of iodination and coupling in vitro. TPO and LPO are equally effective in the selective iodination of a subset of tyrosyl residues, which are able to undergo coupling to form T3 and T4. TPO and LPO also catalyze the coupling of iodothyrosines with similar efficiencies at pH 7.4, even though LPO is significantly less efficient at lower pH (33). Iodine-poor unfractionated hTg, hTg-CS0 and hTg-CS from nonfamilial goiters (with no more than 0.09% of iodine, on a weight basis) were iodinated enzymatically in vitro by using 2 µg/ml of bovine LPO, 7.5 × 10⁻⁵ M potassium iodide, 0.21 µg/ml of glucose oxidase from A. nig er, and 1 × 10⁻³ M D-glucose over 90 min at 25 °C (34). Fig. 3 shows the means ± S.E. of the amounts of iodine incorporated into hTg, expressed as percent of hTg on a weight basis, and of the levels of T3 and T4 formed, as a function of iodine bound during four experiments of time course of iodination in vitro. Each experiment was performed using unfractionated hTg, together with hTg-CS0 and hTg-CS subfractions deriving from the same iodine-poor hTg preparation. The average fraction of hTg-CS in the hTg preparations used was 58.1%. Similar amounts of iodine were incorporated at plateau in hTg and hTg-CS, and slightly higher amounts in hTg-CS0 (Fig. 3, panel A). However, the number of T3 millimoles (panel B) and T4 moles (panel C) synthesized per mol of hTg-CS were in the ratios of 1.70 and 1.34, respectively, with those synthesized per mol of hTg-CS0. Instead, hTg and hTg-CS did not differ, as for the efficiency of formation of T3 and T4. These data indicated that hTg-CS had a higher efficiency of T4 and, especially, T3 formation than hTg-CS0, and that the entire population of unfractionated hTg molecules benefited from this property of hTg-CS. A rationale for this may be that chondroitin 6-sulfate chains exerted an influence not only on the hTg-CS molecules they were linked to but also on the hTg-CS0 molecules they interacted with or on the peroxidase. If so, one would not expect to see...
differences of hormone yields between hTg-CS and hTg-CS$_0$ when these were iodinated altogether in the pool of unfractonated hTg. Such a prediction was verified by the lack of significant variations in the amounts of T3 and T4 formed, between the Q-IEC fractions of hTg preparation O., physiologically iodinated in vivo (Fig. 4). Previous digestion of hTg-CS with 200 milliunits/ml of chondroitinase ABC from P. vulgaris, at pH 8.0 and 37 °C for 4 h, was not associated with reductions of the T3 and T4 yields, with respect to native hTg-CS, and of the differences of hormone-forming efficiency between hTg-CS and hTg-CS$_0$.

**Chondroitin 6-Sulfate Oligosaccharide Unit(s) of hTg-CS Are Restricted in the Carboxy-terminal Region, Downstream Thr2514** — We were able to differentiate hTg-CS from hTg-CS$_0$ in SDS-polyacrylamide gels by staining them with DMTCC (Stains All™, ICN). This dye was reported to stain GAGs metachromatically and, particularly, chondroitin sulfate purple, while staining proteins red (35). In fact, fractions in the Q1 (hTg-CS$_0$) peak were stained pinkish red with DMTCC, and those in the Q2 (hTg-CS) peak were stained purple. Side-by-side comparison of the two peak fractions also revealed a subtle cathodal shift in hTg-CS in the presence of SDS. Both metachromasia and the mobility shift were abolished by digestion with 200 milliunits/ml of chondroitinase ABC, at 37 °C for 4 h, prior to SDS-PAGE (Fig. 5, panel A). To identify the chondroitin 6-sulfate-containing region(s) of hTg, the Q-IEC fractions of hTg were subjected to limited digestion with thermolysin and trypsin, using DMTCC as a probe. The proteolytic fragments, separated by reducing SDS-PAGE, corresponded exactly to those characterized previously by NH$_2$-terminal peptide sequencing (21) and were identified on the basis of their mobilities. Fig. 5, panel B, shows the SDS-PAGE of the digestion products of fractions 29, 40, 48, and 56 of hTg preparation O. with thermolysin. Bands h$_{2TL}$, h$_{5TL}$ and h$_{7TL}$ exhibited metachromasia and a cathodal shift, whose extent was inversely related with the fragment apparent mass, going from fraction 29 in the Q1 peak to fractions 48 and 56 in the Q2 peak. This is in keeping with the notion that peptides with high negative net charges bind lower than average amounts of SDS, and they exhibit lower than average mobilities in SDS-PAGE (36). A mixed pattern was apparent in fraction 40, in the valley between the Q1 and Q2 peaks. Both changes were reverted by digestion of the proteolytic fragments of fraction 48 with chondroitinase ABC, prior to SDS-PAGE, although no changes were brought about in fraction 29 (Fig. 5, panel C). Inspection of the flow-diagram of the limited proteolysis of hTg with thermolysin revealed that fragments h$_{2TL}$, h$_{5TL}$ and h$_{7TL}$ were all located at the carboxyl-terminal side of hTg and shared the region downstream from Leu1832 (Fig. 6, panel A).

On the other hand, a diffuse band (h8-CS$_{TR}$), with an average apparent relative mass of 41,000, appeared among the tryptic fragments of hTg, going from fraction 29 to fraction 40. It was stained purple with DMTCC, but was not apparent in the gel stained with Coomassie Brilliant Blue R-250. Its intensity of staining and apparent relative mass increased going from fraction 40 to 56 (Fig. 5, panel D), in keeping with the increase of D-glucuronic acid content revealed by the analysis of the Q-IEC fractions across the Q2 peak (Fig. 1 and Table 1). Upon digestion of the tryptic fragments of fraction 48 with chondroitinase ABC, band h8-CS$_{TR}$ was replaced by a well focused band, corresponding to dechondroitinated h8-CS$_{TR}$ (h8-deCS$_{TR}$), with an apparent relative mass of 36,000, which was stained blue with Coomassie Brilliant Blue R-250 and red with DMTCC. No changes were caused by chondroitinase ABC in fraction 29 (Fig. 5, panel E). The formation of band h8-deCS$_{TR}$ was not prevented when the limited digestion of hTg-CS with trypsin was preceded, rather than followed, by digestion with chondroitinase ABC under identical conditions. Band h8-deCS$_{TR}$ was prepared by limited tryptic digestion of 0.5 mg of hTg-CS, followed by chondroitinase ABC digestion, reducing SDS-PAGE and transfer to PVDF. NH$_2$-terminal peptide microsequencing of this band revealed a single sequence (TSSKTA), corresponding to hTg residues 2514-2519 (29) (see supplemental Table 1). Naturally, this was also the sequence of peptide h8-CS$_{TR}$. The flow-diagram of limited proteolysis of hTg with trypsin shows the carboxyl-terminal location of peptide h8-CS$_{TR}$, in keeping with the results obtained with thermolysin (Fig. 6, panel B). Because the NH$_2$-terminal sequence of peptide h8-deCS$_{TR}$ was identical with one of two sequences previously found in band h$_{4TR}$ (21), we also determined the sequence of band h$_{4TR}$ prepared from hTg-CS. As expected, two sequences were found, one starting at residue 1 of hTg (h$_{4TR}$) and the other one at residue 2514 (h4bis$_{TR}$) (supplemental Table 1). Thus, of two peptides, both starting at residue 2514, one (h8-CS$_{TR}$) contained chondroitin 6-sulfate oligosaccharide unit(s) and
the other (h4bisTR) did not. Inspection of Fig. 5, panel D, reveals that the increase of staining intensity of band h8-CSSTR, going from fraction 40 to 56, was paralleled by a decrease of intensity of band h4TR, in keeping with the finding that heterodimeric hTg-CS, yielding peptides h8-CSSTR and h4bisTR, and homodimeric hTg-CS, yielding only peptide h8-CSSTR, coexisted in the late portion of the Q2 peak (Fig. 2, panel B).

A Single Type D (Chondroitin 6-Sulfate) Oligosaccharide Unit Is Linked to Ser2730 of hTg-CS - Forty-seven mg of hTg-CS were reduced and carboxymethylated, as reported under “Experimental Procedures”, and hydrolyzed with endoproteinase Glu-C from S. aureus, at the enzyme/substrate weight ratio of 1:100, in 0.05 M sodium phosphate buffer, pH 7.8, at 37 °C for 18 h. Digestion products were subjected to Q-IEC on a 5-ml HiTrap™ Q-Sepharose HP column, using a gradient from 0.1 to 1.2 M NaCl in 0.025 M Tris/HCl, 2.0 M urea, pH 7.4, in 55 min, at the flow rate of 1 ml/min. Most protein was discarded in the flow-through, whereas a unique D-glucuronic acid-containing peak, having negligible absorbance at 280 nm, was eluted late in the gradient (supplemental Fig. 1, panel A). This peak was subjected to size-exclusion chromatography on Bio-Gel P-2 (size-exclusion limit of 1800 relative mass units) in 0.01 M NH₂HCO₃, which yielded a D-glucuronic acid-containing peak in the void volume. Its further purification by size-exclusion chromatography on Sephadex G-50 fine, in 0.01 M NH₂HCO₃, monitored at 220 nm, is shown in supplemental Fig. 1, panel B. Of the two peaks resolved, only one peak contained D-glucuronic acid. NH₂-terminal peptide microsequencing of this material revealed a single, homogeneous nonapeptide with the LTAGXGLRE sequence, corresponding to residues 2726-2734 of the cDNA-derived sequence of hTg (29), X being Ser2730 linked with the chondroitin 6-sulfate oligosaccharide unit (supplemental Table 1). This glycopeptide will be henceforth referred to as hTg-CSgp. The comparison between the sequence around Ser2730 and a consensus for the recognition of serine residues by UDP-D-xyllose:proteoglycan core protein β-D-xyllosyltransferase, derived from the alignment of 51 chondroitin 6-sulfate attachment sites of 19 proteoglycan core proteins (37), revealed a 90% concordance, provided that two insertions of 3 and 2 residues were allowed (Fig. 7). The purified aliquot of hTg-CSgp contained 41.1 µg of D-glucuronic acid and 25.2 µg of protein. On the basis of the M₀ of 903, calculated for the peptide moiety, this indicated an average content of 8.4 mol of D-glucuronic acid per mol of glycopeptide. Electrophoresis of 18 µg of hTg-CSgp in a 16.5% polyacrylamide gel in Tris/Tri/cine/SDS, followed by transfer to a PVDF membrane and staining with DMTCC, revealed a homogeneous, metachromatic band, with the diffuse migration, which is typical of GAGs (17, 32), and an apparent relative molecular mass of 13,000-20,000, both indicating polydispersity (supplemental Fig. 1, panel C). hTg-CSgp was labile upon freezing and thawing.

The Chondroitin 6-Sulfate Oligosaccharide Unit of hTg-CS Protects Peptide Bond Lys2714-Gly2715 from Proteolysis - Next, we sought an explanation for the difference between the apparent relative molecular masses of peptides h8-deCSSTR (M₀, 36,000) and h4bisTR (M₀, 29,000), by determining the carboxyl terminus of the latter. In fact, only a minor part of the mass difference could be accounted for by the residual oligosaccharide (GlcUA-Gal-Gal-Xyl), which was left in situ after chondroitinase ABC digestion. Even though peptide h4bisTR comigrated with h4TR, in reducing SDS-PAGE of the products of tryptic digestion of hTg described above, previous observations indicated that the homologous peptide b11TR of bovine Tg was free of comigrating species, under nonreducing conditions, because it was the sole fragment not linked to other fragments by disulfide bonds (38). In fact, nonreducing SDS-PAGE of the tryptic fragments of hTg yielded only two bands instead of several bands seen with reduction (supplemental Fig. 2, panel B). NH₂-terminal microsequencing of band h4TR-NR, transferred onto PVDF, revealed the same sequence as peptide h4bisTR, starting at residue 2514, together with traces of a sequence starting at residue 2518 (supplemental Table 1). Thus, the products of limited tryptic digestion of goatier hTg (with an iodine content of 0.03%, on a weight basis), at the enzyme/substrate weight ratio of 1:100, at 30 °C for 20 min, were subjected to reverse-phase HPLC in the absence of reduction, using a Vydac C-4 column (250 × 10 mm, 5 µm) and a gradient of acetonitrile in trifluoroacetic acid (supplemental Fig. 2, panel A). Nonreducing SDS-PAGE of peak 2 revealed band h4TR-NR. Upon reduction, this band exhibited the same apparent relative mass as peptide h4bisTR (supplemental Fig. 2, panel B). The mass values associated with this band were determined by ES-MS (supplemental Table 2) and defined a peptide having ragged
amino- and carboxyl-terminal ends (residues 2512-2514 and 2713-2714, respectively) and containing a high-mannose oligosaccharide unit, composed of 2 N-acetyl-D-glucosamine and 8 or 9 D-mannose residues, linked to Asn2563, as reported (7, 29, 39). Furthermore, the ES-MS analysis of HPLC peak 1 revealed a mass value corresponding to hTg peptide 2715-2745 (supplemental Table 2). Thus, peptide h4bisTR was truncated at Lys2714, whereas peptide bond Lys2714-Gly2715 was protected from proteolysis in peptide h8-CS TR, which extended through Ser2730, with its bound chondroitin 6-sulfate unit. Another tryptic site occurred between Lys2745 and Thr2746 (Fig. 7).

DISCUSSION

The present study shows that type D (chondroitin 6-sulfate) oligosaccharide units are a main source of molecular microheterogeneity of hTg, being regularly found in a significant and sometimes predominant fraction of it. We developed an IEC method, which permitted us to separate chondroitin 6-sulfate-containing hTg molecules (hTg-CS) from the residual hTg molecules (hTg-CS0). By exploiting the changes of electrophoretic mobility and staining properties conferred upon the products of limited proteolysis of hTg by chondroitin 6-sulfate units, we first restricted the chondroitin 6-sulfate-containing regions of hTg to a carboxyl-terminal peptide, starting at Thr2514. The subsequent purification of a homogeneous, D-glucuronic acid-containing nonapeptide (hTg-CSgp), corresponding to hTg residues 2726-2734, permitted us to establish Ser2730 as the sole site of chondroitin 6-sulfate addition in hTg. The surrounding sequence showed concordance with a consensus sequence for the recognition of core protein serine residues by UDP-D-xylose:proteoglycan core protein β-D-xylosyltransferase, with the exception of two insertions of three and two residues (Fig. 7), in keeping with the finding that recognition sequences of proteins, which are not modified quantitatively with chondroitin sulfate, match the consensus less well than those of proteins that are (37). The presence in hTg of O-linked oligosaccharide units, composed of 1 mol of D-xylose, 2 mol of D-galactose, 11 mol of a D-glucuronic acid-N-acetyl-D-galactosamine disaccharide, and up to 14 mol of sulfate per mol of serine was reported (8). Based on numbers of moles of D-glucuronic acid per mol of hTg varying from 4.8 to 8.7 in different hTgs, it was suggested that some hTg molecules might be devoid of type D units, although some might contain units of different sizes, or multiple units (8). Subsequent studies showed that the repeating disaccharides were of the chondroitin 6-sulfate type (9, 10). Our data show that a relevant hTg fraction regularly contained a single chondroitin 6-sulfate unit per polypeptide chain, linked to Ser2730 and composed of a broadly varying number of repeating disaccharide units. In some goiter hTg preparations with high contents of hTg-CS, hTg-CS heterodimers, in which only one monomer contained a chondroitin 6-sulfate chain, coexisted with hTg-CS homodimers. In the monomers derived from the dissociation of hTg-CS in urea, the net number of disaccharide units per chondroitin 6-sulfate chain did not exceed 14.

We also show that hTg-CS had a higher efficiency of hormone formation than hTg-CS0 and that the whole unfractionated hTg benefitted from this property. Thus, chondroitin 6-sulfate addition represents an ergonomic mechanism, by which the post-translational modification of a fraction of molecules influences the overall function of hTg. The greater advantage in the formation of T3, with respect to T4, associated with the chondroitin 6-sulfate unit, and the proximity of the attachment site of the latter to the carboxyl terminus of hTg suggest that it may influence hormonogenesis by affecting the function of the site of preferential T3 formation at Tyr2747 (29, 40, 41). Normal ranges of serum T4 concentration (64 to 142 nmol/liter) and T3 concentration (1.1 to 2.9 nmol/liter) have higher limits that exceed the lower ones only by a factor of 2.2 and 2.6, respectively (42), so that the increases in the rates of T4 and T3 formation in hTg-CS, in comparison with hTg-CS0, observed in the present study may be physiologically significant. The regulation of the T4/T3 ratio in thyroid secretion is crucial for maintaining physiological concentrations of active hormone (T3) in blood and is finely tuned by multiple mechanisms, under the control of TSH (2). The T4/T3 ratio is regulated first at the biosynthetic level in Tg and decreases in experimental animals given TSH. In rabbit and guinea pig Tg, TSH stimulates T3 formation at tyrosine 2747, and decreases T4 synthesis at tyrosine 5 (40). The diminished T4 formation at the amino terminus of Tg is mediated by the TSH-stimulated maturation of the N-linked oligosaccharide chain linked to Asn91, from the high-mannose to the complex type (3). The data that we present contribute to delineate a common mechanism, mediated by
modifications of the composition and number of N-linked and O-linked oligosaccharide chains, which may be responsible for changes in the hormonogenic efficiency at both hTg termini, affecting the overall ratio of T4 over T3 formation in hTg. For hormone release to occur, Tg must be internalized into thyroid follicular cells and degraded in lysosomes. A part of Tg, internalized via the endocytic receptor megalin, bypasses lysosomes and is transcytosed across cells, to be secreted from the basolateral pole (43). Tg binding to megalin is facilitated by accessory interactions with heparan-sulfate proteoglycans on the surface of thyroid cells (44, 45). Experiments are in progress to determine whether chondroitin 6-sulfate units may interfere with the binding of hTg to megalin. Conceivably, the addition of chondroitin 6-sulfate to hTg, besides improving the yield of active hormone, may also prevent the nonproductive internalization of hTg by transcytosis.

Furthermore, we identified two sites, one between Lys2714 and Gly2715 and the other between Lys2745 and Thr2746, near the site of chondroitin 6-sulfate addition, which were susceptible to limited proteolysis with trypsin. Our data show that the former was protected from proteolysis in hTg-CS but not in hTg-CS0. Both the hormone-forming advantage of hTg-CS over hTg-CS0 and the resistance of peptide bond 2714-2715 to proteolysis persisted after digestion of hTg-CS with chondroitinase ABC. Such findings indicate that the residual oligosaccharide that was left in situ was sufficient for the observed effects. This is not surprising with respect to proteolysis, because O-linked mono- and disaccharides were able to protect specific cleavage sites from cathepsin L (12). Moreover, the production of proteolytic fragments by an oxidative cleavage mechanism was repeatedly observed in the course of Tg iodination both in vitro (46, 47) and in vivo (48). The protection from the occurrence of such cleavages in the carboxyl-terminal end of hTg, possibly afforded by the oligosaccharide chain at Ser2730, even in its residual form after digestion with chondroitinase ABC, might contribute to improve the hormone yields. Verification of this hypothesis will be the aim of future work. It is worth noticing that the product of digestion of hTg-CS with chondroitinase ABC differed from native hTg-CS0, because this enzyme hydrolyzes the β-(1→4)-glycosidic bonds between N-acetyl-D-galactosamine 6-sulfate and D-glucuronic acid, thus removing β-GlcUA-(1→3)-GalNAc-6S disaccharides, while leaving in situ a GlcUA-Gal-Gal-Xyl linkage oligosaccharide. Moreover, Hascall et al. (49) reported that the disaccharide closest to the linkage oligosaccharide in chondroitin 4-sulfate resisted digestion by chondroitinase ABC. Our data entail, even though some of the hTg molecules in the Q1 peak might contain short oligosaccharide chains of this kind, that all the hTg molecules from the Q2 peak digested with chondroitinase ABC contained residual stubs, in a heterodimeric or even in a homodimeric form.

The reported effects on proteolysis are in support of a possible modifying influence of the chondroitin 6-sulfate unit in the processing of hTg by APCs, and in the ability of hTg to function as an autoantigen, particularly because the chondroitin 6-sulfate unit was located within an epitope-rich region, harboring several T cell-related epitopes, capable of causing EAT in genetically susceptible mice, and B cell-related epitopes, recognized by circulating autoantibodies of patients with AITD (reviewed in Ref. 1). The effects of oligosaccharide chains on the activity of proteolytic enzymes involved in antigen processing and on the affinity of epitope binding to major histocompatibility complex or T cell receptor molecules have been reviewed (11). O-Linked mono- and disaccharides in tumor-associated glycoprotein MUC1 restricted the repertoire of the epitopes produced and/or presented in a site-specific manner, either by limiting the proteolytic accessibility of the protein (12) or by preventing epitope recognition by a peptide-specific T cell hybridoma (13). N-linked oligosaccharide chains inhibited the generation of a self-epitope from glutamate receptor subunit 3 (14) and of cytotoxic lymphocyte-specific epitopes from influenza A nucleoprotein (15). Moreover, a keratan sulfate chain masked an arthritogenic T cell epitope in the G1 domain of aggrecan, whereas the depletion of multiple chondroitin sulfate side chains generated clusters of chondroitin sulfate stubs, which activated specific B cells to function as APCs (16). We expect that the chondroitin 6-sulfate chain of hTg may inhibit the processing of hTg peptide 2731-2744 (amino acid numbering as per Ref. 29), known for its ability to stimulate in vitro strong proliferative responses and the adoptive transfer of EAT by splenic lymphocytes of CBA mice immunized with mouse Tg (50). Other epitopes might be abrogated by the chondroitin 6-sulfate unit, should their generation require
cleavage in the vicinity of Ser2730, within a range including the Lys2714-Gly2715 peptide bond on the amino side.

In addition, significant effects of chondroitin sulfate oligosaccharide chains on cellular immune responses have been documented. A small percentage (2-5%) of invariant chain molecules, associated with class II major histocompatibility complex molecules, are modified with the addition of a single chondroitin sulfate chain at Ser291 (II-CS). In this form, they remain associated with class II molecules at the surface of APCs (51, 52), where they act as accessory molecules in antigen presentation, facilitating the interactions between APCs and T cells, and greatly enhancing class II-dependent allogeneic and mitogenic T cell responses (17). Such effects occur through interactions of II-CS with CD44 on responding T cells, as they can be inhibited both by anti-CD44 antibodies, and by a soluble form of CD44 (CD44Rg), which binds II-CS directly (17). Treatment of spleen cells with xyloside, which inhibits GAG addition (53), interferes with their antigen-presenting capabilities (54). Serglycins, small proteoglycans stored in secretory granules of hematopoietic cells, activate the CD3-dependent release of cytokines and proteases from CD44-positive cytotoxic lymphocytic clones (18) by interacting with CD44 through their chondroitin 4-sulfate and 6-sulfate side chains (19). Moreover, CD44 binding to aggregcan, a major proteoglycan of the cartilage matrix, through the chondroitin 4- and 6-sulfate side chains of the latter, can trigger the oligomerization of CD44 molecules and the activation of intracellular signaling (20). In keeping with these suggestions, preliminary results indicate that the chondroitin 6-sulfate oligosaccharide unit significantly affects the immunopathogenic capacity of hTg in CBA/J(H-2A^d) mice by a complex mechanism.2

Finally, the close clinical association between Graves hyperthyroidism and thyroid-associated ophthalmopathy (TAO) led to the hypothesis that the latter might be the result of an autoimmune response against orbital autoantigen(s) that are also present in the thyroid. Candidate antigens, besides the TSH receptor, include Tg (55). Some authors detected hTg in the orbital tissues of patients with TAO and hypothesized that it might be transferred there via thyroid-orbit connections evidenced by lymphography (56). hTg was found prevalently in fibroadipose tissue (57). It was proposed that the ability of hTg to bind to GAGs, including chondroitin sulfate B and C, might mediate its localization in orbital tissues, where it might function as a target of immune responses directed against the thyroid (58). Metachromatic GAGs accumulate in TAO and thyroid-associated dermopathy (55). Edematous connective perimysial tissues of patients with TAO are composed mainly of hyaluronan and chondroitin sulfate (59). Accumulation of GAGs and adipose tissue expansion is apparent in the fatty connective tissue of the posterior orbit (60). In our opinion, the presence of an integral GAG chain, in a fraction of hTg molecules, may represent a mechanism by which autoimmune responses against hTg may spread to connective tissue antigens with shared GAG chains, particularly in the event that the synthesis of both be quantitatively and/or qualitatively dysregulated. Proving this hypothesis will require the fine structural characterization of the chondroitin 6-sulfate chains of hTg and of the GAGs of orbital connective tissues from patients with TAO, and the demonstration of cross-reacting B and/or T cell clones.

In conclusion, a single chondroitin 6-sulfate chain linked to Ser2730 in a relevant fraction of hTg molecules influences both the hormone-forming efficiency, and the proteolytic accessibility of the carboxyl-terminal region of hTg. These effects may bear consequences on thyroid homeostasis and autoimmunity. Further work will be aiming to determine the following: 1) What are the physiological limits of hTg-CS abundance in hTg? 2) How is the synthesis of the chondroitin 6-sulfate unit of hTg regulated, particularly regarding the role of TSH? May changes of hTg-CS abundance mediate thyroid adaptation to iodine deficiency or inherited defects of thyroid hormone synthesis or secretion? 3) Do any correlations exist between thyroid function and variations in the hTg-CS/hTg-CSa ratio or in the chondroitin 6-sulfate chain length, as analyzed by gel electrophoresis of the oligosaccharide units released from hTg by β-elimination, in comparison with appropriate standards? 4) How does the chondroitin 6-sulfate unit influence the processing of hTg by APCs and cellular immune responses to hTg in vivo? It is our opinion that a systematic investigation may shed light on the pathogenesis of thyroid diseases, particularly AITD.
REFERENCES

1. Gentile, F., Conte, M., and Formisano, S. (2004) *Immunology* **112**, 13-25
2. Gentile, F., Di Lauro, R., and Salvatore, G. (1995) in *Endocrinology* (DeGroot, L. J. ed) 3rd Ed., Vol. 1, pp. 517-542, W. B. Saunders Co., Philadelphia
3. Mallet, B., Lejeune, P. J., Baudry, N., Niccoli, P., Carayon, P., and Franc, J. L. (1995) *J. Biol. Chem.* **270**, 29881-29888
4. Fenouillet, E., Fayet, G., Hovsepian, S., Bahraouï, E. M., and Ronin, C. (1986) *J. Biol. Chem.* **261**, 15153-15158
5. Arima, T., Spiro, M. J., and Spiro, R. G. (1972) *J. Biol. Chem.* **247**, 1825-1835
6. Arima, T., and Spiro, R. G. (1972) *J. Biol. Chem.* **247**, 1836-1848
7. Yang, S.-X., Pollock, G., and Rawitch, A. B. (1996) *Arch. Biochem. Biophys.* **327**, 61-70
8. Spiro, M. J. (1977) *J. Biol. Chem.* **252**, 5424-5430
9. Schneider, A. B., McCurdy, A., Chang, T., Dudlak, D., and Magner, J. (1988) *Endocrinology* **122**, 2428-2435
10. Spiro, R. G., and Bhoyroo, V. D. (1988) *J. Biol. Chem.* **263**, 14351-14358
11. Anderton, S. M. (2004) *Curr. Opin. Immunol.* **16**, 753-758
12. Hanisch, F.-G., Schwientek, T., Von Bergwelt-Baildon, M. S., Schultze, J., and Finn, O. (2003) *Eur. J. Immunol.* **33**, 3242-3254
13. Vlad, A., Müller, S., Cudic, M., Paulsen, H., Otvos, L., Hanisch, F.-G., and Finn, O. J. (2002) *J. Exp. Med.* **196**, 1435-1446
14. Gahring, L., Carlson, N. G., Meyer, E. L., and Rogers, S. W. (2001) *J. Immunol.* **166**, 1433-1438
15. Wood, P. and Elliott, T. (1998) *J. Exp. Med.* **188**, 773-778
16. Glant, T. T., Buzás, E. I., Finnegan, A., Negroiu, G., Cs-Szabó, G., and Mikecz, K. (1998) *J. Immunol.* **160**, 3812-3819
17. Naujokas, M. F., Morin, M., Anderson, M. S., Peterson, M., and Miller, J. (1993) *Cell* **74**, 257-268
18. Toyama-Sorimachi, N., Sorimachi, H., Tobita, Y., Kitamura, F., Yagita, H., Suzuki, K., and Miyasaka, M. (1995) *J. Biol. Chem.* **270**, 7437-7444
19. Toyama-Sorimachi, N., Kitamura, F., Habuchi, H., Tobita, Y., Kimata, K., and Miyasaka, M. (1997) *J. Biol. Chem.* **272**, 26714-26719
20. Fujimoto, T., Kawashima, H., Tanaka, T., Hirose, M., Toyama-Sorimachi, N., Matsuzawa, Y., and Miyasaka, M. (2001) *Int. Immunol.* **13**, 359-366
21. Gentile, F., and Salvatore, G. (1993) *Eur. J. Biochem.* **218**, 603-621
22. Palumbo, G., Tecce, M. F., and Ambrosio, G. (1982) *Anal. Biochem.* **123**, 183-189
23. Blumenkrantz, N., and Asbøe-Hansen, G. (1973) *Anal. Biochem.* **54**, 484-489
24. Spiro, R., (1966) *Metth. Enzymol.* **8**, 3-25
25. Warren, L. (1963) *Meth. Enzymol.* **6**, 463-464
26. Hollander, C. S., and Shenkman, L. (1974) In Rothfeld, B. (ed.): *Nuclear medicine in vitro*, Lippincott, Philadelphia, 136-149
27. Wessol, D., and Flügge, U. I. (1984) *Anal. Biochem.* **138**, 141-143
28. Dahlberg, A. E., Dingman, C. W., and Peacock, A. C. (1969) *J. Mol. Biol.* **41**, 139-147
29. van de Graaf, S. A. R., Ris-Stalpers, C., Pauws, E., Mendive, F. M., Targovnik, H. M., and de Vijlder, J. J. M. (2001) *J. Endocrinol.* **170**, 307-321
30. Schägger, H., and von Jagow, G. (1987) *Anal. Biochem.* **166**, 368-379
31. Gentile, F., Veneziani, B. M., and Sellitto, C. (1997) *Anal. Biochem.* **244**, 228-232
32. Malsch, R., Harenberg, J., Piazolo, L., Huhle, G., and Heene, D. L. (1996) *J. Chromatogr. B Biomed. Appl.* **685**, 223-231
33. Deme, D., Pommier, J., and Nunez, J. (1978) *Biochim. Biophys. Acta* **540**, 73-82
34. Lamas, L., Santisteban, P., Turmo, C., and Seguido, A. M. (1986) *Endocrinology* **118**, 2131-2136
35. Bader, J., Ray, D. A., and Steck, T. L. (1972) *Biochim. Biophys. Acta* **264**, 73-84
36. Pitt-Rivers, R., and Impiombato, F. S. (1968) *Biochem. J.* **109**, 825-830
37. Brinkmann, T., Weilke, C., and Kleessiek, K. (1997) *J. Biol. Chem.* **272**, 11171-11175
38. Veneziani, B. M., Giallauria, F., and Gentile, F. (1999) *Biochimie* **81**, 517-525
39. Rawitch, A. B., Liao, T. H., and Pierce, J. G. (1968) *Biochim. Biophys. Acta* **160**, 360-367
40. Fassler, C. A., Dunn, J. T., Anderson, P. C., Fox, J. W., Dunn, A. D., Hite, L. A., Moore, R. C., and Kim, P. S. (1988) *J. Biol. Chem.* **263**, 17366-17371
41. Lamas, L., Anderson, P. C., Fox, J. W., and Dunn, J. T. (1989) *J. Biol. Chem.* **264**, 13541-13545
42. Larsen, P. R., Davies, T. F., Schlumberger, M.-J., and Hay, J. D. (2003) in *Williams Textbook of Endocrinology* (Larsen, P. R., Kronenberg, H. M., Melmed, S., and Polonsky, K. S., eds) 10th Ed., pp. 331-373, W. B. Saunders Co., Philadelphia

43. Marinò, M., Zheng, G., Chiovato, L., Pinchera, A., Brown, D., Andrews, D., and McCluskey, R. T. (2000) *J. Biol. Chem.* **275:** 7125-7138

44. Marinò, M., Friedlander, J. A., McCluskey, R. T., and Andrews, D. (1999) *J. Biol. Chem.* **274:** 30377-30386

45. Marinò, M., Andrews, D., and McCluskey, R. T. (2000) *Thyroid* **10:** 551-559

46. Dunn, J. T., Kim, P. S., and Dunn, A. D. (1982) *J. Biol. Chem.* **257:** 88-94

47. Dunn, J. T., Kim, P. S., Dunn, A. D., Heppner, D. G. Jr., and Moore, R. C. (1983) *J. Biol. Chem.* **258:** 9093-9099

48. Duthoit, C., Estienne, V., Delom, F., Durand-Gorde, J.-M., Mallet, B., Carayon, P., Ruf, J. (2000) *Endocrinology* **141:** 2518-2525

49. Hascall, V. C., Riolo, R. L., Hayward, J. Jr., and Reynolds, C. C. (1972) *J. Biol. Chem.* **247:** 4521-4528

50. Hoshioka, A., Kohno, Y., Katsuki, T., Shimo, N., Maruyama, N., Inagaki, Y., Yokochi, T., Tarutani, O., Hosoya, T., and Niimi, H. (1993) *Immunol. Lett.* **37:** 235-239

51. Sant, A. J., Cullen, S. E., and Schwartz, B. D. (1985) *J. Immunol.* **135:** 416-422

52. Miller, J., Hatch, J. A., Simonis, S., and Cullen, S. E. (1988) *Proc. Natl. Acad. Sci. USA* **85:** 1359-1363

53. Fogelfeld, L., and Schneider, A. (1990) *Endocrinology* **126:** 1064-1069

54. Rosamond, S., Brown, L., Gomez, C., Braciale, T., and Schwartz, B. (1988) *J. Immunol.* **139:** 1946-1951

55. Prabhakar, B. S., Bahn, R. S., and Smith, T. J. (2003) *Endocrine Rev.* **24:** 802-835

56. Marinò, M., Lisi, S., Pinchera, A., Mazzi, B., Latrofà, F., Sellari-Franceschini, S., McCluskey, R. T., and Chiovato, L. (2001) *Thyroid* **11:** 177-185

57. Lisi, S., Marinò, M., Pinchera, A., Mazzi, B., Di Cosmo, C., Sellari-Franceschini, S., and Chiovato, L. (2002) *Thyroid* **12:** 351-360.

58. Marinò, M., Lisi, S., Pinchera, A., Marcoceci, C., Menconi, F., Morabito, E., Macchia, M., Sellari-Franceschini, S., McCluskey, R. T., and Chiovato, L. (2003) *Thyroid* **13:** 851-859

59. Kahaly, G., Forester, G., and Hansen, C. (1998) *Thyroid* **8:** 429-432

60. Hufnagel, T. J., Hickey, W. F., Cobbs, W. H., Jakobiec, F. A., Iwamoto, T., and Eagle, R. C. (1984) *Ophthalmology* **91:** 1411-1419

**FOOTNOTES**

* This work was supported by PRIN 2004 Grant 2004062075 from the Ministero dell’Istruzione, Università e Ricerca, Rome, Italy (to F.G.). The costs of publication of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: AITD, autoimmune thyroid disease; APC, antigen-presenting cell; DMTCC, 3,3’-diethyl-9-methyl-4,5,4’,5’-dibenzothiacarbocyanine; EAT, experimental autoimmune thyroiditis; ES-MS, electrospray mass spectrometry; GAG, glycosaminoglycan; h8-DCS18, peptide h8-DCS18 digested with chondroitinase ABC; HPLC, high pressure liquid chromatography; hTg, human thyroglobulin; hTG-CS, type D (chondroitin 6-sulfate) oligosaccharide unit-containing hTg; hTG-CS0, type D (chondroitin 6-sulfate) oligosaccharide unit-devoid hTg; hTG-CSgp, chondroitin 6-sulfate-containing glycopeptide 2726-2734 of hTg; li, invariant chain; li-CS, chondroitin sulfate-containing invariant chain; LPO, lactoperoxidase; MMI, 2-mercapto-1-methylimidazole; PVDF, polyvinylidene difluoride; Q-IEC, ion-exchange chromatography on trimethylamino-substituted Q-Sepharose; T3, 3,5,5’-triiodothyronine; T4, 3,5,3’,5’-tetraiodothyronine (thyroxine); TAO, thyroid-associated ophthalmpathy; Tg, thyroglobulin; TPO, thyroid peroxidase; TSH, thyrotropin.

2 Conte, M., Arcaro, A., D’Angelo, D., Gnata, A., Fulciniti, F., Formisano, S. and Gentile, F., manuscript in preparation.
FIGURE LEGENDS

Figure 1. Fractionation by ion-exchange chromatography on HiTrap™ Q-Sepharose HP (Q-IEC) of selected hTg preparations, and compositional analysis of the resulting fractions. Twenty-mg aliquots of hTg preparation O. (panel A), Ma. (panel B), and D. (panel C), in 0.025 M Tris/HCl, 0.05 M NaCl, pH 7.4, were loaded onto a 5-ml HiTrap™ Q-Sepharose HP column, equilibrated with 0.025 M Tris/HCl, pH 7.4 (buffer A). After washing with buffer A, a linear gradient from 0 to 100% of buffer B (1.2 M NaCl in buffer A), in 24 min, at the flow rate of 2.5 ml/min, was applied (the dashed-dotted line indicates the NaCl concentration at the column head, with reference to the left y axis; dead volume was 6.0 ml). One-mL fractions were monitored for protein content, by measuring the optical absorbance at 280 nm (continuous line), and carbohydrate content. The symbols used are as follows: neutral hexoses content, in mmol/liter (hatched line); D-glucuronic acid (GlcUA) content, in μmol/liter (dashed line) and mol/hTg mol (●); N-acetyleneuraminic acid (NANA) content, in mol/hTg mol (○). The analysis of one fraction every other two by PAGE under native conditions, in a 4-9% total acrylamide gradient gel stained with Coomassie Brilliant Blue R-250, is shown on top of panels A and B.

Figure 2. Fractionation by Q-IEC of selected hTg preparations, followed by Q rechromatography of the peaks obtained therefrom, under various conditions. Panel A, following the Q-IEC of 20 mg of hTg preparation I., as described in the legend to Fig. 1, the fractions of the Q1 and Q2 peaks obtained therefrom were pooled separately, with the exclusion of three fractions between peaks. Each pool was dialyzed against buffer A and subjected to Q rechromatography, under identical conditions as the initial Q-IEC. The optical absorbance at 280 nm of the fractions of the initial Q-IEC (continuous line) and of the Q rechromatography of the Q1 (dotted line) and Q2 peak (dashed line) was monitored. Panel B, following the Q-IEC of 20 mg of hTg preparation Mi. N., the fractions of the Q2 peak were pooled, dialyzed against buffer A, dissociated in 0.04 M Tris/HCl, 2.5 M urea, pH 9.0, at 20 °C overnight, as reported (31), and subjected to Q rechromatography by using a NaCl gradient from 0 to 1.2 mol/liter in 2.5 M urea, 0.055 M Tris/HCl, pH 7.4. The symbols used are as follows: optical absorbance at 280 nm of the fractions of the initial Q-IEC (continuous line) and of the Q rechromatography of the Q2 peak dissociated in urea (dashed line); D-glucuronic acid (GlcUA) content, in mol/hTg mol, of the fractions of the initial Q-IEC (●) and of the Q rechromatography of the Q2 peak dissociated in urea (○). Panel C, following the Q-IEC of 20 mg of hTg preparation Ca., the fractions of the Q2 peak were pooled. An aliquot of 6 mg of this material was taken to the volume of 1.5 ml with Centriprep 30 concentrators, dialyzed against 0.1 M Tris, 0.1 M sodium acetate, pH 8.0, digested with 200 milliunits/ml of chondroitinase ABC from P. vulgaris for 4 h at 37 °C, and finally subjected to Q rechromatography. The optical absorbance at 280 nm of the fractions of the initial Q-IEC (continuous line) and of the Q rechromatography of the Q2 peak digested with chondroitinase ABC (dotted line) was monitored.

Figure 3. Enzymatic in vitro iodination and analysis of the hormone-forming efficiency of hTg, hTg-CS₈ and hTg-CS. Iodine- and hormone-poor hTg (●) and its subfractions hTg-CS₈ (■) and hTg-CS (○), prepared by Q-IEC, at the concentration of 0.45 g/liter in 0.02 M imidazole/HCl, pH 7.0, were iodinated enzymatically in vitro, using 2 μg/ml of bovine LPO, 7.5 × 10⁻⁵ M potassium iodide, 0.21 μg/ml of glucose oxidase from A. niger and 1 × 10⁻³ M D-glucose, over 90 min at 25 °C (34). Individual experiments were performed using unfractonated hTg, hTg-CS₈, and hTg-CS subfractions derived from the same iodine-poor hTg preparation. The average percent content of hTg-CS in the hTg preparations used was 58.1%. At the times indicated, aliquots were removed, dialyzed against 0.01 M NH₄HCO₃, 0.005 M NaCl, and the iodine content was assayed and expressed as percent of the protein on a weight basis (panel A). After digestion with Pronase and aminopeptidase M, as described under “Experimental Procedures”, T3 (panel B) and T4 (panel C) were also assayed by radioimmunoassay. Points represent mean ± S.E. values of four experiments. For the sake of clarity, S.E. values are indicated only for hTg-CS₈ and hTg-CS.

Figure 4. Iodine and hormone content of the Q-IEC fractions of physiologically iodinated hTg preparation O., from normal thyroid tissue, containing 0.50% iodine (w/w). The hTg preparation was subjected to Q-IEC, as described in the legend to Fig. 1, and the fractions obtained were analyzed for their iodine (▲), T3 (●) and T4 (○) content, as described under “Experimental Procedures”. The
continuous line indicates the optical absorbance at 280 nm, for which measuring units are not indicated as they were the same as in Fig. 1.

Figure 5. SDS-PAGE under reducing conditions of selected Q-IEC fractions of hTg preparation O., either as such or subjected to limited proteolysis with thermolysin or trypsin and/or digestion with chondroitinase ABC. Panel A, top fractions of the Q1 peak (fraction 29) and Q2 peak (fraction 48), analyzed before (-) and after (+) digestion with 200 milliunits/ml of chondroitinase ABC, in a 4-13% total acrylamide gradient gel stained with DMTCC. The positions of chondroitinase ABC (ABC) and carrier bovine serum albumin (BSA) are indicated. Panel B, digestion products of the fractions indicated with thermolysin (TL), at the TL/hTg ratio of 1:50, pH 7.8, and 30 °C for 80 min, analyzed in replicate 4-17% total acrylamide gels, stained with Coomassie Brilliant Blue R-250 (left) and DMTCC (right). Fragments are marked at right in accordance with Ref. 21. Fragments exhibiting cathodal shifts and metachromasia, going from the Q1 to the Q2 peak, are shown in purple. Panel C, further digestion with chondroitinase ABC of the products of proteolysis of fractions 29 and 48 with thermolysin. Panel D, digestion products of the fractions indicated with trypsin (TR), at the TR/hTg ratio of 1:100, pH 7.8 and 30 °C for 40 min. Panel E, digestion with chondroitinase ABC of the products of proteolysis of fractions 29 and 48 with trypsin. Dechondroitinated peptide h8-CS_TR (h8-deCS_TR) is circled. Relative molecular mass standards (Sf), as detailed in “Experimental Procedures”, are marked at left of each gel.

Figure 6. Flow-diagrams of the limited proteolysis of hTg with thermolysin (panel A) and trypsin (panel B) (21). Each bar represents a peptide, with the peptide number above the bar, the apparent relative molecular mass below at right and the amino-terminal residue number below at left, according to Ref. 29. Fragments of hTg-CS, which exhibited cathodal shifts and metachromasia upon staining with DMTCC, with respect to their counterparts deriving from hTg-CSb, were drawn gray. Their apparent relative molecular masses, both before and after digestion with chondroitinase ABC (ABC), are indicated. Cross-hatching of bars h4TL and h2TR indicates that, under the digestion conditions used, the corresponding peptides were not detected in the gels shown in Fig. 5.

Figure 7. Diagrammatic representation of the localization and structure of type D (chondroitin 6-sulfate) oligosaccharide unit of hTg. The cDNA-deduced amino acid sequence of hTg, from residue 2710 to residue 2749, is represented in single-letter code (29). Black circles with white lettering mark the sequence found for the purified glycopeptide hTg-CSgp (with the proviso that Ser2730 appeared as a blank). A consensus sequence for the recognition of core protein serine residues by UDP-D-xylose:proteoglycan core protein β-D-xylosyltransferase (37) is aligned with the hTg sequence, and concordances are indicated. A diagram of the chondroitin 6-sulfate oligosaccharide unit attached to Ser2730 is shown (8-10). The number of repeating β-D-glucuronic acid-N-acetyl-D-galactosamine 6-sulfate disaccharide units per chondroitin 6-sulfate chain found in the present study is indicated. The abbreviations used are as follows: Xyl, D-xylose; Gal, D-galactose; GlcUA, D-glucuronic acid; GalNAc, N-acetyl-D-galactosamine. The two sites of tryptic cleavage between residues 2714-2715 and 2745-2746 found in the present study and the preferential site of T3 formation at Tyr2747 (29, 40, 41) are also marked.
TABLE I – Compositional analysis of selected hTg preparations subjected to Q-IEC on HyTrap™ Q-Sepharose HP

| hTg preparation<sup>a</sup> | hTg-CS, % of hTg | D-Glucuronic acid in Q2 peak | Total neutral hexoses | NANA | Iodine, % of hTg (w/w) |
|---------------------------|------------------|-----------------------------|----------------------|------|----------------------|
|                           |                  | Total, mol/hTg mol<sup>b</sup> | Range<sup>c</sup>, mol/hTg mol | Range<sup>d</sup>, % (w/w) | Range<sup>d</sup>, mol/hTg mol | |
| O.                        | 65.8             | 11.8                        | 10.0                 | 3.8-14.3 | 10.7-10.2 | 18.0-19.8 | 0.50 |
| Ma.                       | 38.9             | 3.9                         | 7.0                  | 3.9-13.2 | 8.0-10.4 | 15.1-18.2 | 0.12 |
| T.                        | 48.3             | 7.2                         | 7.7                  | 3.7-14.0 | 4.9-6.1 | 9.8-9.9 | 0.02 |
| Ce.                       | 51.2             | 7.5                         | 7.3                  | 2.8-14.2 | 7.0-8.5 | - | 0.09 |
| D.                        | 66.3             | 14.8                        | 11.7                 | 3.9-15.7 | 4.4-5.4 | 16.0-14.0 | 0.16 |
| Mi.                       | 71.6             | 20.9                        | 15.6                 | 3.1-23.8 | - | - | 0.16 |
| Mi. N. <sup>e</sup>       | 63.1             | 16.8                        | 12.0                 | 3.6-24.0 | - | - | 0.08 |
| Mi. N. Q2 peak in urea<sup>f</sup> | 72.7         | 20.2                        | 13.6                 | 3.2-14.8 | - | - | - |

<sup>a</sup> hTg preparations were named after the patients (using the last initial(s)). Preparation O. was from a euthyroid individual, hemilaryngectomized for a non-thyroidal disease; the other preparations were from non-familial, simple, or multinodular goiters.

<sup>b</sup> Values were measured in the fractions with the maximal protein concentration in the Q2 peak.

<sup>c</sup> Data indicate the D-glucuronic acid concentration range across the span of the Q2 peak, starting from the valley between the Q1 and the Q2 peaks.

<sup>d</sup> Data indicate the concentration range across the entire span of the Q-IEC, from the leading edge of the Q1 peak to the trailing edge of the Q2 peak.

<sup>e</sup> Preparation obtained from a large 2 × 3-cm colloid nodule, surrounded by apparently normal thyroid tissue, from patient Mi.

<sup>f</sup> Pooled fractions of the Q2 peak from the Q-IEC of hTg preparation Mi. N. were dissociated in 0.04 M Tris/HCl, 2.5 M urea, pH 9.0, for 18 h at 20 °C, as reported (31), and then subjected again to Q-IEC, using a gradient from 0 to 1.2 M NaCl in 0.055 M Tris/HCl, 2.5 M urea, pH 7.4.
SUPPLEMENTAL DATA

FIGURE LEGENDS

Supplemental Figure 1. Isolation and purification of the chondroitin 6-sulfate-containing glycopeptide hTg-CSgp. Panel A, the products of digestion of 47 mg of carboxymethylated hTg-CS with endoproteinase Glu-C were loaded onto a 5-ml HiTrap™ Q-Sepharose HP column, in 0.025 M Tris/HCl, 2.0 M urea, pH 7.4, containing 0.1 M NaCl, and eluted with a gradient from 0.1 to 1.2 M NaCl in the same buffer (the dashed line indicates the NaCl concentration at the column head, with reference to the left y axis). One-ml fractions were monitored for protein content, by measuring their optical absorbance at 280 nm (continuous line), and D-glucuronic acid (GlcUA) content (dotted line). Panel B, size-exclusion chromatography on Sephadex G-50, in 0.01 M NH₄HCO₃, of the D-glucuronic acid-containing peak eluted in the void volume of the size-exclusion gel chromatography on Bio-Gel P-2 (not shown) of the D-glucuronic acid-containing fractions of the Q-IEC shown in panel A; one-ml fractions were monitored for peptide content, by measuring their optical absorbance at 220 nm (continuous line), and D-glucuronic acid (GlcUA) content (dotted line). Panel C, Analysis of 18 µg of glycopeptide hTg-CSgp, by electrophoresis in a 16.5% total acrylamide gel in Tris/Tricine/SDS (30), and electrophoretic transfer to a PVDF membrane, stained with DMTCC. Relative molecular mass standards (St, myoglobin cleavage products and glucagon, as detailed under “Experimental Procedures”) are marked at right.

Supplemental Figure 2. Isolation of carboxyl-terminal tryptic fragments of hTg. Panel A, reverse-phase HPLC of the nonreduced products of limited digestion of 1 mg of goiter hTg with trypsin, at the enzyme/substrate weight ratio of 1:100, at pH 7.8 and 30 °C, for 20 min, using a Vydac C-4 column (250 × 10 mm, 5 µm) equilibrated in 0.1% (v/v) trifluoroacetic acid in dH₂O (solvent A), containing 2% of 0.07% trifluoroacetic acid in acetonitrile (solvent B). The column was developed with the gradient of solvent B indicated by the dotted line (see under “Experimental Procedures” for details). One-ml fractions were collected and monitored for peptide content, by measuring their optical absorbance at 220 nm (continuous line). The main peaks are numbered. Panel B, SDS-PAGE analysis of the total tryptic digestion products of hTg (Tot), and of peaks 2 and 3 of the HPLC shown in panel A, in a 4-17% total acrylamide gradient gel, under reducing and nonreducing conditions, as indicated. Bands h4ₜ and h4ₜ NR are indicated. Relative molecular mass standards (St) are marked at right.
Figure 3

A

Iodine % in hTg, w/w

Incubation time, min

B

$T_3$, mmoles / hTg mole

Iodine % in hTg, w/w

C

$T_4$, moles / hTg mole

Iodine % in hTg, w/w
Figure 7

Tryptic site

2715 G K A G D

2720 A

2725 G Q S A A

2730 E E E E E E E L T A G S G L R E D L L S Q

2735 K S Y T K S G P E

2740 Tryptic site

2745 K S Y T K S G P E

Consensus:

a a a a a G S G a b a a

(a: E, D; b: G, E, D)

GalNAC GlcUA (SO3)

Gal

Xyl

Gal

SO3

n = 3 - 14
A single chondroitin 6-sulfate oligosaccharide unit at Ser2729 of human thyroglobulin enhances hormone formation and limits proteolytic accessibility at the carboxyl terminus. Potential insights into thyroid homeostasis and autoimmunity

Marisa Conte, Alessia Arcaro, Daniela D'Angelo, Ariele Gnata, Gianfranco Mamone, Pasquale Ferranti, Silvestro Formisano and Fabrizio Gentile

J. Biol. Chem. published online May 5, 2006 originally published online May 5, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M513382200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2006/06/09/M513382200.DC1