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ScienceDirect
Life Sciences 80 (2007) 538–545
Life Sciences
www.elsevier.com/locate/lifescie

Presence of β-linked GalNAc residues on *N*-glycans of human thyroglobulin

Akira Takeya [a,*], Osamu Hosomi [b], Hironori Nishijima [a], Yoshihide Ohe [c], Kunio Sugahara [d], Morihisa Sagi [a], Kentaro Yamazaki [a], Hideyuki Hayakawa [a], Hiroshi Takeshita [a], Chizuko Sasaki [a], Tadahisa Kogure [a], Toshiji Mukai [a]

[a] *Department of Legal Medicine, St. Marianna University School of Medicine, 1-16-2 Sugao, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan*
[b] *Department of Health Science, School of Health and Sports Science, Juntendo University, Hiragagakuendai, Inba-mura, Chiba 270-1695, Japan*
[c] *Biosignal Research Center, Institute for Molecular and Cellular Regulation, Gunma University, Showa-machi, Maebashi, Gunma 371-8511, Japan*
[d] *Department of Bioproductive Science, Faculty of Agriculture, Utsunomiya University, Mine-machi, Utsunomiya, Tochigi 321-8505, Japan*

Received 6 January 2006; accepted 5 October 2006

Abstract

Hepatic asialoglycoprotein receptor, which may mediate the clearance of circulating thyroglobulin, is known to have a high affinity for GalNAc. Recently, the receptor has been reported to be present also in the thyroid, implicating interaction with thyroglobulin. Here, mammalian thyroglobulins were analyzed for GalNAc termini by Western blotting with GalNAc-recognizing lectins labeled with peroxidase or ^{125}I. *Wistaria floribunda* lectin was found to bind human thyroglobulin and, to some extent, bovine, but not porcine thyroglobulin. After desialylation, the lectin bound all of the thyroglobulins tested. The binding was inhibited by competitive inhibitor GalNAc. Peptide *N*-glycanase treatment of human desialylated thyroglobulin resulted in the complete loss of reactivity with *W. floribunda* lectin, indicating that the binding sites are exclusively on *N*-glycans. The binding sites on human desialylated thyroglobulin were partly sensitive to β-galactosidase, and the remainder was essentially sensitive to β-*N*-acetylhexosaminidase. On the other hand, the binding sites of bovine and porcine desialylated thyroglobulins were totally sensitive to β-galactosidase. Thus the lectin binds β-Gal termini, as well as β-GalNAc. GalNAc-specific *Dolichos biflorus* lectin also bound human thyroglobulin weakly. In contrast to *W. floribunda* lectin, desialylation diminished binding, suggesting that these two lectins recognize different GalNAc-terminated structures. Again, the binding was inhibited by GalNAc and by treatment with peptide *N*-glycanase. These results strongly indicate the presence of distinct GalNAc termini of *N*-glycans on human thyroglobulin.

Keywords: *Wistaria floribunda* lectin; *Dolichos biflorus* lectin; *N*-glycan; *N*-acetylgalactosamine; Thyroglobulin; Thyroid

Introduction

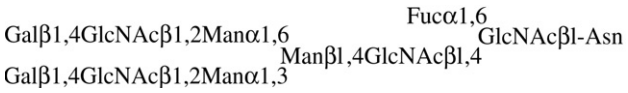
Thyroglobulin (Tg), the precursor of thyroid hormone, is synthesized by thyrocytes and secreted into the lumen of thyroid follicles, where it is stored as the major colloidal component. Moreover, Tg is iodinated, and hormone synthesis occurs with both thyroid peroxidase and the H_2O_2 generating system, mainly in the follicular lumen. Hormone release requires the uptake of Tg from the colloid by thyrocytes, and proteolytic cleavage along the lysosomal pathway (Dunn and Dunn, 2000).

Glycosylation is one of major post-translational modifications of Tg and is considered to play a multifaceted role in thyroid hormonogenesis and regulation (Deshpande and Venkatesh, 1999). For examples, it regulates the secretion of Tg (Björkman and Ekholm, 1982), binding of molecular chaperones, which aid in the folding and matulation of Tg (Kim et al., 1992), targeting of Tg to subcellular compartments (Consiglio et al., 1981; Pacifico et al., 2003), iodine content and hormonogenesis (Bastiani et al., 1995; Mallet et al., 1995), and antigenicity of Tg (Salabè et al., 1976; Fenouillet et al., 1986), and so on.

The *N*-glycans of human, bovine and porcine Tgs (hTg, bTg and pTg, respectively) are composed mainly of both high mannose and complex types (Spiro, 1965). The complex-type

* Corresponding author. Tel.: +81 44 977 8111; fax: +81 44 977 3902.
E-mail address: a2takeya@marianna-u.ac.jp (A. Takeya).

0024-3205/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2006.10.004



Scheme 1. A typical, complex-type *N*-glycan of Tg (Yamamoto et al., 1981; De Waard et al., 1991). The Gal residues are often substituted with sialic acid or sulfate.

glycans are typically diantennary (Scheme 1) (Fukuda and Egami, 1971; Yamamoto et al., 1981, 1984; De Waard et al., 1991). The Gal residues are often capped with sialic acid or sulfate to form mainly NeuAc/Gcα2,6Gal, and occasionally NeuAc/Gcα2,3Gal, or SO_4–3Gal sequences. The triantennary structure is also reported. In addition, hTg has two types of *O*-glycans. One consists of a chondroitin sulfate chain containing a Gal and Xyl linkage region (Spiro, 1977). The other is, perhaps, GalNAcα1-Ser/Thr-based (Arima et al., 1972), but is not well characterized. To date, no GalNAc-terminated structure, nor its sialylated form, on *N*-glycans is reported.

Some Tg is diverted from colloid to the bloodstream by thyrocytes. Two types of cells, hepatocytes and macrophages, are considered to be involved in the clearance of circulating Tg. Hepatocyte asialoglycoprotein receptor is located on the cell surface, binds a variety of plasma asialoglycoproteins carrying Gal and GalNAc termini, and mediates endocytosis with the ligands (Ashwell and Harford, 1982). It has a much higher affinity for GalNAc than for Gal (Lee and Lee, 1987). The recent study indicated that the receptor exhibits an affinity for NeuAcα2,6GalNAc-R also, but not for NeuAcα2,6Gal-R (Park et al., 2003). Thus, Tg carrying GalNAc or NeuAcα2,6GalNAc termini, if present, might be more easily trapped by hepatocytes to be degraded. The residual Tg is trapped by macrophages (Brix and Herzog, 1994), which carry mannose receptors on their cell surface. The macrophages are antigen-presenting cells and might also function in the autoimmune response. Moreover, asialoglycoprotein receptor has recently been demonstrated to be present at the apical membrane of thyrocytes, indicating that the receptor functions in uptake of Tg from colloid to hormone release (Pacifico et al., 2003). Hence, GalNAc or sialylated GalNAc termini would facilitate the uptake of Tg.

Here, we explore terminal GalNAc residues of some native, or desialylated, mammalian Tgs using several GalNAc-recognizing lectins. All desialylated and, sometimes, even the native Tgs tested bind to both *Wistaria floribunda* lectin (WFL) and soybean lectin (SBL), but the binding sites on Tgs are mostly sensitive to β-galactosidase. Only hTg carries WFL- or SBL-binding sites sensitive to β-*N*-acetylhexosaminidase. It is also shown that hTg carries *Dolichos biflorus* lectin (DBL)-binding sites.

Materials and methods

Materials

Samples of purified hTg were purchased from Biogenesis (UK) for hTg-1 and -2 and from Calbiochem (USA) for hTg-3. Purified bTg samples were obtained from Sigma (USA) and Wako (Japan), and those of purified pTg were from Sigma and Serva (Germany), called in order Tg-1 and -2, respectively. Gels (4–20% gradient polyacrylamide) for electrophoresis were from Daiichi Pure Chemicals (Japan). Immun-Blot and Sequi-Blot polyvinylidene difluoride (PVDF) filters (0.2 μm pore size) and biotinylated SDS-PAGE standards were from Bio-Rad (USA). The following horseradish peroxidase (HRP)-labeled lectins were purchased: WFL and *Helix pomatia* lectin (HPL) were from EY Laboratories (USA), and SBL, DBL and Gal-specific *Ricinus communis* lectin (RCL-I) were from Wako. Rabbit anti-human thyroglobulin antibody (N1565) and HRP-labeled porcine anti-rabbit immunoglobulin antibody were from Dako (Denmark). 3,3′-Diaminobenzidine (DAB) was from Amresco (USA). *Arthrobacter ureafaciens* sialidase was from Nacalai Tesque (Japan). Jack bean β-*N*-acetylhexosaminidase and *Streptococcus* 6646 K β-galactosidase were from Seikagaku (Japan). Chicken liver α-*N*-acetylgalactosaminidase and bovine serum albumin (A3059; BSA) were from Sigma. *Flavobacterium meningosepticum* peptide *N*-glycanase (PNGase) (Tarentino et al., 1985) was from Takara (Japan).

Western and dot blots

Tg samples were subjected to 4–20% gradient SDS-PAGE in reducing conditions (Laemmli, 1970), and electroblotted onto PVDF filters (Towbin et al., 1979). In some experiments, samples were directly applied to filters in a circle of 3 mm in diameter. The filters were stained with Coomassie Brilliant Blue (CBB), or blocked with 1% BSA in 50 mM Tris–HCl-buffered saline, pH 7.4, and incubated with 5 μg/ml lectin conjugated with HRP for 1 h at 4 °C, followed by 500 μg/ml of DAB and 0.01% hydrogen peroxide (Hawkes, 1982). In sugar inhibition experiments, 200 mM GalNAc was added to solutions containing labeled lectins.

In exoglycosidase digestion experiments, filters blocked with BSA were treated at 37 °C with the following glycosidases, in a total volume of ~125 μl containing 0.02% NaN_3 as preservative, prior to addition of HRP-lectins: 0.125 U of *A. ureafaciens* sialidase in 0.5 M acetate buffer, pH 5.0 (Uchida et al., 1979), 1.5 U of jack bean β-*N*-acetylhexosaminidase (Li and Li, 1970) or 0.3 U of chicken liver α-*N*-acetylgalactosaminidase in 0.3 M citrate–phosphate buffer, pH 5.0, and 0.01 U of *S.* 6646 K β-galactosidase in 0.1 M acetate buffer, pH 5.5, containing 10 mM $MnCl_2$ (Kiyohara et al., 1976). As controls, enzymes were treated at 100 °C for 5 min to be inactivated, and used as above.

In some experiments, filters blocked with BSA were incubated as primary antibody with ready-to-use anti-hTg antibody, followed by 1000-fold diluted, HRP-labeled second antibody. Negative control (non-immune rabbit antibody as primary antibody) gave no positive reaction.

^{125}I-labeled lectin

Non-labeled WFL (Wako) was dissolved (1 mg/ml) in 50 mM Tris–HCl-buffered saline, pH 7.4. It was radiolabeled by a modification of an Iodogen-catalyzed reaction (Fraker and Speck, 1978), according to the limit of daily usage