A Mutation Causing Reduced Biological Activity and Stability of Thyroxine-Binding Globulin Probably as a Result of Abnormal Glycosylation of the Molecule

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T_4-binding globulin (TBG), a 54-kilodalton glycoprotein, is the major thyroid hormone transport protein in man. The exact nature of the mutations causing X chromosome-linked TBG deficiency, which affect about 1 in 2,500 newborn males, is unknown. Here we report the sequence of a unique variant TBG (TBG-Gary) encoding a protein with severely impaired T4 binding as well as decreased stability at 37 C, resulting in its rapid *in vivo* denaturation. A single nucleotide substitution in the codon for residue 96 of the mature protein replaces isoleucine with asparagine; this replacement creates an additional site for N-linked glycosylation. The anodal shift of TBG-Gary on isoelectric focusing gel electrophoresis suggests that this new site is likely glycosylated. Since glycosylation is required for TBG to assume its correct tertiary structure, but is not subsequently necessary for maintenance of the biological properties or stability of the molecule, we believe that the likely presence of additional carbohydrate probably affects a higher order structure of the molecule and is thus responsible for the reduced stability and hormone binding activity of TBG-Gary (TBG^{ASN-96}) (Molecular Endocrinology 3: 575–579, 1989)

INTRODUCTION

T_4-binding globulin (TBG), is a 54 kilodalton serum glycoprotein composed of a single polypeptide chain and four carbohydrate residues (1, 2). It is synthesized by the liver (3, 4), and its principal function is to bind thyroid hormone and thus, increase its intravascular pool (2). Changes in TBG concentration produce proportional alterations in the level of T_4 in serum (5–8). However, because the hormone is transported into cells in a free rather than TBG-bound form, TBG abnormalities have no effect on the metabolic state, general health, or survival of the individual (1, 2, 5–8). Nevertheless, inappropriate therapeutic interventions to correct the alterations of thyroid hormone concentration in the serum of such individuals are not uncommon, and in fact, can be detrimental (8, 9) This is particularly relevant as inherited abnormalities of TBG are relatively common, affecting about 1 of 2,500 newborn males (10, 11). Inherited TBG defects are X chromosome linked and expressed in hemizygotes as complete or partial TBG deficiency or as TBG excess (2, 5–8). Heterozygous females usually have TBG values intermediate to those of affected and unaffected males.

We recently described an unusual family with low serum thyroid hormone concentrations due to TBG deficiency (12). The disorder was recognized fortuitously during the investigation of the proband for short stature which proved to be due to Turner's syndrome (female phenotype with a single X chromosome). Studies revealed a unique TBG which was expressed in the proband and other members of the family (TBG-Gary), which had greatly reduced affinity for T_4 that was 100-fold lower than normal (Refetoff, S., unpublished), as well as decreased stability resulting in its rapid *in vivo* denaturation (12). As a consequence, specific RIAs detected a decrease in the concentration of the abnormal native protein (nTBG) and an increase of denatured TBG (dnTBG) in the sera of the affected subjects.

We now present the results of cloning and sequencing this variant gene which revealed a T→A transversion in the coding region resulting in the replacement of Ile-96 with an Asn. This replacement creates a new site

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for N-linked glycosylation. As TBG-Gary appears to have additional carbohydrate, we believe that its presence is likely responsible for the altered properties of this protein, probably through changes in the conformation of the molecule.

RESULTS AND DISCUSSION

A complete pedigree of living members of this family with their respective nTBG and dnTBG levels in serum is shown on Fig. 1. The nTBG concentrations in the two hemizygous affected subjects [the proband (III-1) and her father (II-2)] are 1.5% the mean normal level, while those of dnTBG are approximately 10-fold above the normal mean. Two heterozygous subjects [paternal grandmother (I-1) and sister (III-2)] have nTBG and dnTBG values intermediate between those of hemizygous-affected and unaffected subjects. Thus, the inheritance of TBG-Gary is clearly X-linked, as expected from the recent demonstration of a single TBG gene copy located on the X chromosome (13).

The 4.5-kilobase pair (kbp) TBG gene is contained within a 14 kbp *Eco*Ri fragment and is composed of 4 protein coding exons (Fig. 2). The gene of the affected father (II-2, see Fig. 1) of the proband was isolated, and the sequences of these 4 exons were compared to those of a normal human liver TBG cDNA clone and a genomic TBG clone from a human X chromosome library (14) as well as 3 genomic clones from an individuals with the common type TBG (Mori, Y., and S. Refetoff, unpublished). The only difference between these 6 sequences was a T→A transversion in the first protein coding exon of the TBG-Gary gene, 287 bp

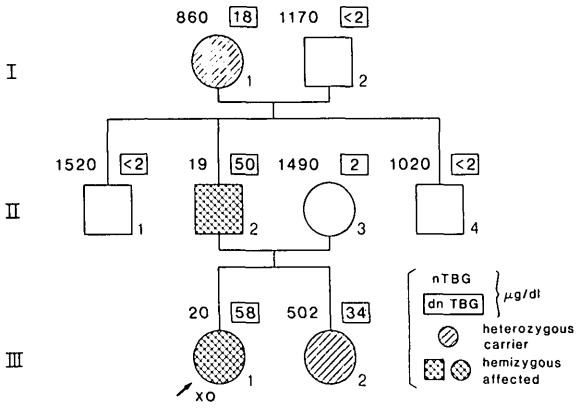

Fig. 1. Pedigree of the Family Expressing TBG-Gary with Values of Native TBG (nTBG) and Denatured TBG (dnTBG)

Concentrations expressed in micrograms per dl are shown on the *left* of each symbol for nTBG and, *boxed* in on the *top right* of each symbol, for dnTBG. Mean normal values ± 1 SD are 1530 ± 180 for nTBG and 6.1 ± 2.3 for dnTBG. *Roman numerals* indicate generations and *numbers* on the *bottom right* of each symbol identify individual subjects. Note that the proband (III-1, indicated by the *arrow*), has a single X chromosome (XO-Turner's syndrome).

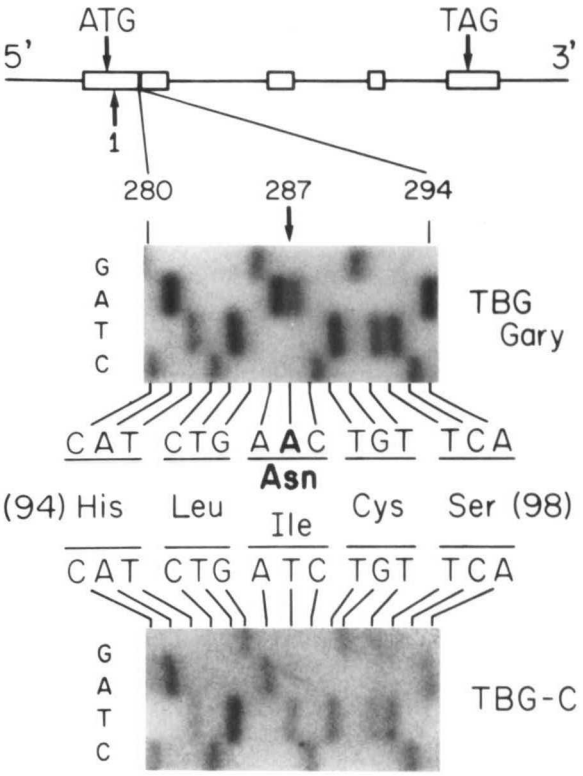

Fig. 2. Structure of the TBG Gene and Sections of Sequencing Gels Showing the Mutation Site in TBG-Gary Compared to the Corresponding Sequence of the Common Type TBG (TBG-C)

In the diagram of the TBG gene, exons are represented by *open boxes* and intervening and flanking sequences by lines. Nucleotide 287, from the cordon for the NH_2-terminus of the mature protein, is a T in TBG-C and A in TBG-Gary. Note that the resulting substitution of amino acid-(96) creates a potential N-linked glycosylation site (Asn-Cys-Ser). The amino acids at the intron splice sites are: intron-1, Ala-188 (G|CC); intron-2, Gly-279 (GG|A); and intron-3, Asn-328 (AAT|).

downstream of the starting point of the mature protein, resulting in the replacement of the normal Ile-96 codon (ATC) by Asn (AAC) (Fig. 2). This amino acid substitution, which was verified by sequencing both sense and antisense strands, creates a potential site of glycosylation (Asn-Cys-Ser). Moreover, the nucleotide substitution destroys the recognition site for the restriction enzyme *Sau*3AI (GATC→GAAC), which allowed us to relate the TBG abnormality in individual family members to the presence of the mutation.

This exon, from four key members of the family, was amplified using the polymerase catalyzed chain reaction and then was digested with *Sau*-3AI. As shown in Fig. 3, the two affected hemizygous subjects (II-2 and III-1) differ from their normal unaffected relative (II-3) and an unrelated individual, expressing TBG-C, by having a 416 bp fragment, rather than one of 394 bp fragment, due to the inability of *Sau*3AI to cleave off a 24 bp fragment from the TBG-Gary gene. As expected, the heterozygous subject (III-2) has both 418 bp and 394 bp fragments. Thus, it seemed very likely that the TBG

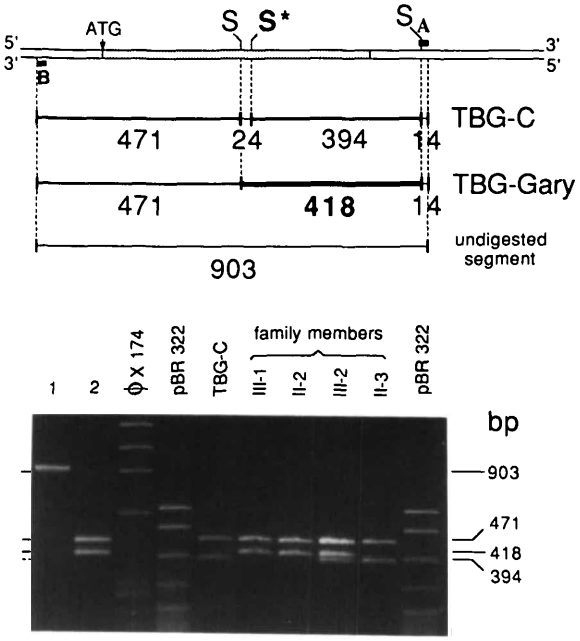

Fig. 3. Detection of the TBG-Gary mutation by *Sau*3AI digestion of amplified DNA sequences

The strategy of analysis is shown diagrammatically on the *upper* portion of the figure. The coding sequences of the first exon (*stippled area*) is contained in the amplified 903 bp fragment having at each end sequences corresponding to the two primers (A and B, see *Materials and methods*). The positions of the *Sau*3AI sites (S) and the expected sizes of fragments generated after digestion of TBG-C and TBG-Gary alleles are indicated (*bars and numbers*). The mutation of TBG-Gary resulting in the loss of the recognition site for *Sau*3AI (S*) generates a 418 bp (*bold bar*) rather than the normal 394 bp fragment generated by TBG-C. The 24 bp and 14 bp fragments are too small for visualization on the gel.

Lanes 1 and 2, Cloned TBG-Gary gene, undigested and after digestion, respectively; ϕX 174 and pBR 322, markers digested with *Hae*III and *Msp*I, respectively; TBG-C, an unrelated subject expressing TBG-C, III-1 and II-2, hemizygous subjects expressing TBG-Gary (see Fig. 1); III-2, heterozygous subject expressing both TBG-C and TBG-Gary (see Fig. 1); II-3, mother of III-1 and III-2, expressing TBG-C (see Fig. 1).

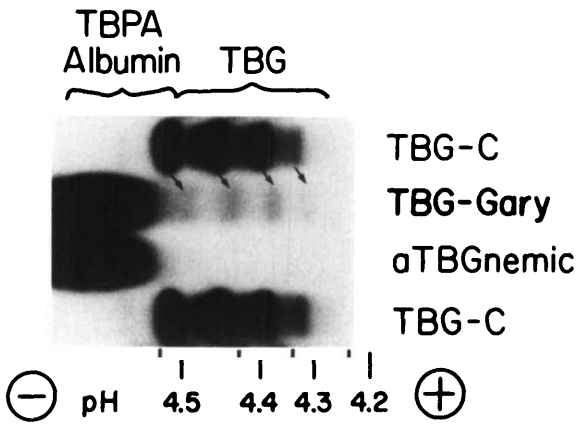

Fig. 4. IEF Analysis of TBG-Gary

Serum samples from two unrelated individuals with the common type TBG (TBG-C), from a subject with TBG-Gary (II-2, see Fig. 1) and a subject with complete TBG deficiency (aTBGnemic) were incubated with $[^{125}I]T_4$ then submitted to IEF and radioautography. The four distinct bands with pI values ranging from 4.28 to 4.48 represent the normal microheterogeneous forms of TBG-C, each with decreasing sialic acid content. All four bands of TBG-Gary are shifted anodally (*arrows*) in accordance with an increased sialic acid content. No $[^{125}I]T_4$ localized to the TBG zone in the serum from the aTBGenemic subject. Note that the wide band of ^{125}I activity cathodal to pH 4.5 represents $[^{125}I]T_4$ bound to secondary binding sites in prealbumin and albumin due to either the reduced T_4-binding affinity of TBG-Gary or absence of TBG in the aTBGenemic serum.

abnormality observed in this family is due to the synthesis of TBG^{Asn-96}.

To determine whether the new potential site of glycosylation was indeed glycosylated, we exploited the observation that isoelectric focusing (IEF) can be used to reveal microheterogeneity in serum TBG resulting from variation in sialic acid content; addition of sialic acid reduces the isoelectric point (pI) of the TBG heteromolecule (15). The uniform anodal shift of all TBG-Gary bands (Fig. 4) is consistent with the presence of additional sialic acid in all heteromolecules, suggesting that Asn-96 of TBG-Gary is likely glycosylated.

Since no amino acid replacements other than the substitution of Asn for Ile at position 96 were found in TBG-Gary, it is likely that the abnormal properties of this protein are the consequence of this mutation. Although the Ile for Asn change is relatively conservative, we cannot exclude a direct effect of this amino acid substitution on T_4 binding and TBG stability. We believe that it is more likely that the properties of TBG-Gary are due to the presence of the additional carbohydrate, possibly by altering the secondary or tertiary structure of the protein. This interpretation is supported by our previous observation that while glycosylation is required for TBG to assume its tertiary structure before secretion, the complete removal of carbohydrates from the mature secreted protein does not alter its biological or immunological properties (16, 17).

Since carbohydrate moieties are often required for the proteolytic or conformational stability of the protein component of glycoproteins (18), we searched for other examples of naturally occurring mutations resulting in a loss or gain of Asn. Of the seven such mutations reported in man (19–24), none resulted in the elimination or creation of the canonical recognition signal, Asn-Xaa-Ser/Thr, for N-linked glycosylation (25). One of these mutations, a substitution of Ser-406 for Asn in the immunoglobulin μ heavy chain, created the sequence Thr-Phe-Asn which surprisingly appears to be more highly glycosylated (22). The molecule is defective in initiating complement-dependent cytolysis. Santos-Aguado *et al.* (26) studied the functional role of carbohydrates in the α-chain of the major histocompatibility complex class I antigen by site-directed mutagenesis. The elimination of the unique glycosylation site on Asn-86, by its substitution with either Gln or Asp, lowered

or abolished expression of the molecule on the surface of transfected cells but retained most antigenic determinants. The functional defect of the molecule resulting from these mutations was attributed to conformational changes of the α-chain and its interaction with the β_2-microglobulin.

We believe that this may be the first description of a natural mutation that results in the synthesis and secretion of an apparently abnormally glycosylated protein having altered biological properties. Finally, we would also like to propose changing the eponym, TBG-Gary, to TBGc$^{Asn\text{-}96}$ which better describes this first variant TBG to be characterized in molecular terms.

MATERIALS AND METHODS

Subjects and TBG Analyses

Blood was obtained from members of the family (see Fig. 1) for the identification of the variant TBG in their serum and for the isolation of DNA from white blood cells. Native TBG and dnTBG concentrations were measured by specific RIAs with the lowest level of detectability being 2 μg/dl (27). IEF analysis of TBG was carried out as previously described (26) using a horizontal slab gel containing 5% acrylamide and 6.7% ampholines, covering a pH range of 4.2 to 4.9. Five microliters of sera from two subjects having normal concentrations of the common type TBG (TBG-C) and 32 μl sera from two unrelated individuals expressing the variant TBG in low concentrations (TBG-Gary) or having complete TBG deficiency (aTBGnemic), were equilibrated, at room temperature, with 10 nCi [^{125}I]T_4 (SA, 1250 μCi/μg, New England Nuclear, Boston, MA) and applied to the prefocused gel. After completion of the IEF run, the gel was dried and radioautographed on X-AR5 film (Eastman Kodak, Rochester, NY). Note that application to the gel of 32 μl of the normal serum diluted 60-fold with serum from the aTBGnemic subject, to adjust the concentration of TBG-C to that in serum of subjects with TBG-Gary, did not alter its IEF mobility.

Cloning and Sequencing

DNA, purified from white blood cells by the method of Madisen *et al.* (29), was digested with *Eco*RI, as directed by the manufacturer (Bethesda Research Laboratories, Gaithersburg, MD). Fragments of 10–20 kbp, which are enriched for the TBG gene, were isolated by electrophoresis in a low melting ajarose gel (30) and ligated into *Eco*RI arms of λEMBL-4. After packaging and infection of *Escherichia coli* strain LE 392, clones containing the TBG gene were identified by hybridization with the insert from human liver cDNA clone (λcTBG-8, 14) labeled with [^{32}P]deoxycytidine trisphosphate by nick translation kit (Amersham, Arlington Heights, IL) according to the instructions of the manufacturer. Appropriate restriction fragments were subcloned into M13 mp18 or mp19 and sequenced by the dideoxy chain termination method (31).

DNA Amplification, Restriction Endonuclease Digestion, and Analysis by Electrophoresis

The DNA segment of the entire sequence of the first coding exon containing the mutation site was amplified using the polymerase catalyzed chain reaction (32, 33(B). Oligonucleotides (20mers), 5′-CCCTGATGAGCACATCATCA-3′ corresponding to the 5′-intron sense sequence (B) and 5′-CAGTGGAGCAGATCACTGTG-3′ corresponding to 3′-intron antisense sequence (A) served as primers (Fig. 3). The predicted size of the amplified segments of genomic DNA from members of the family was verified by electrophoresis in 2.0% NuSieve GTG agarose gel (FMC BioProducts, Rockland, ME) and visualized with UV light after staining with ethidium bromide. Amplified DNA segments were digested with *Sau*3AI (Bethesda Research Laboratories, Gaithersburg, MD), and fragments were resolved by electrophoresis in 2.0% NuSieve agarose gel as previously described (33).

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