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Original paper

CARBOHYDRATE CHARACTERIZATION OF PURIFIED THYROXINE-BINDING GLOBULIN BY LECTIN BLOT AND ISOELECTRIC FOCUSING

Ivana Petrović, Svetlana Savin, Ivona Baričević, Dubravka Cvejić

Institute for the Application of Nuclear Energy INEP, Zemun

Summary: The structure of carbohydrate moiety of purified thyroxine-binding globulin (TBG) was examined by lectin blot and isoelectric focusing (IEF). In lectin blot, TBG reacted positively with the following lectins: *Sambucus nigra* agglutinin (SNA I), *Ricinus communis* agglutinin (RCA I), wheat germ lectin (WGA), phytoheamagglutinin (PHA) and pea lectin (PSA). The obtained results indicate that purified TBG contains N-linked oligosaccharide chains consisting of mannose, galactose, N-acetylglucosamine and sialic acid. Isoelectric focusing of TBG at pI 4.2 4.6 revealed three bands, which confirmed that isolated TBG had retained its structure without desialylation. Lectin blot analysis and IEF can be considered to be useful tools in the study of TBG glycosylation.

Key words: thyroxine - binding globulin (TBG), glycosylation, lectins, isoelectric focusing

Introduction

Thyroxine-binding globulin (TBG) is the major carrier molecule for transporting thyroid hormones in human serum. It is an acidic glycoprotein of hepatic origin with total carbohydrate content varying from 15 to 20% of weight. The proposed carbohydrate composition of TBG comprises four biantennary and triantennary branched, asparagine-linked oligosaccharide side chains: glycopeptide I, oligosaccharide A (two chains) and oligosaccharide B. They consist of mannose, galactose, N-acetylglucosamine and N-acetylneuraminic (sialic) acid in the molar ratios 12:9:17:9, respectively (1, 2). The terminal sialic acid residues (6 10) are partly responsible for the microheterogeneous behavior of TBG molecule upon isoelectric focusing (IEF). Namely, three to four major and several minor bands were obtained within the pI range 4.1 4.9 (3 5).

Study of the carbohydrate structure of TBG molecule usually employed chemical methods (1, 2). In recent years, lectin affinity methods have become a very useful tool in carbohydrate studies and several attempts have been made to reveal sugar variances in carbohydrate moiety of TBG (6 9).

We have recently obtained a purified TBG preparation from pregnant women sera (10). In the current study its carbohydrate structure was examined using lectin affinity blot. Supplementary glycoside characterization of purified TBG was obtained after isolectric focusing (IEF) on thin layer polyacrylamide gel.

Material and Methods

Material

TBG preparation was isolated from pregnant women sera by a two step chromatographic procedure as previously described (10).

Na ^{125}I was obtained from the Institute of Isotopes Corporation (Budapest, Hungary). Pharmalyte Ampholine (pI 4.2 4.9) was purchased from Pharmacia Biotech (Vienna, Austria). Horseradish peroxidase type VI and Ricinus lectin were products of ICN Biochemicals (Cleveland, Ohio). LT_2 was purchased from Sigma Chemical Co.

$^{125}IT_4$ (SA specific activity: 1350 μCi/μg) was prepared by labelling LT_2 with 5 mCi of Na^{125}I in our laboratory according to the method of Hunter and Greenwood using chloramine-T (11).

Address for correspondence

mr Ivana Petrović
Institute for the Application of Nuclear Energy INEP
Banatska 31b
11080 Zemun
e-mail: ivanap@inep.co.yu

XP X-ray film (Curix, 13 × 18 cm) was purchased from AGFA (Germany), X-ray fixer and X-ray developer were products of Borex (RTB Bor, Serbia).

All other chemicals used in this study were p.a. grade.

Methods

SDS - PAGE was performed in 12.5% gel (12).

Lectin blot: The following plant lectins, with different carbohydrate specificities, were conjugated with horseradish peroxidase (HPRO) in our laboratory: phytohaemagglutinin (PHA) isolated from *Phaseolus vulgaris* (13), wheat germ lectin (WGA) purified from *Triicum vulgare* (14), pea lectin (PSA) isolated from *Pisum sativum* (15) and *Sambucus nigra* agglutinin (SNA I) (16). Ricinus communis agglutinin (RCA I) was commercially obtained. The pure TBG preparation (50 μg) obtained from pregnant women sera (1) was separated by SDS - PAGE (12) and transfered to nitrocellulose membranes (NC) by semi-dry electrotransfer as previously described (17). NC membranes, SDS acrylamide gels and filter papers were previously equilibrated in blotting buffer 0.025 mmol/L Tris-HCl (pH 8.3), 0.192 mol/L glycine, 20% (v/v) methanol. After electrotransfer, NC membrane was incubated with 3% (w/v) bovine serum albumin in 0.05 mol/L Tris-HCl buffer (pH 7.6) containing 0.15 mol/L NaCl (TBS) overnight at 4 °C. NC was incubated with lectin-HRPO conjugates for 2h at room temperature (18). They were used in the following dilutions in TBS: PHA 1:1000, WGA 1:1000, PSA 1:50, SNA I 1:500 and RCA I 1:2000. After washing with TBS-Tween, diaminobenzidine and urea-peroxide were added to develop the colour reaction.

For isoelectric focusing (IEF), 0.2 mm thick polyacrylamide gels were made on Gel Bond containing 3.96 mL of acrylamide (10% T and 0.3% C), 0.528 mL of Pharmalyte pI 4.2 4.9 (final concentration 6.6% w/v) and 13.2% of glycerol. Polymerization was achieved by adding 2.6 μL N,N,N',N'-tetramethylethylenediamine (TEMED) and 72.7 μL 2.28% w/v ammonium persulfate (APS); 1 mol/L H_3PO_4 and 0.2 mol/L NaOH were used as electrode solutions for the anode and cathode, respectively. Gels were prefocused at 3000 V, 30 mA, 2W at 8 °C up to 350 V/h to generate a pI gradient of 4.2 4.9. Serum samples (20 μL), previously incubated with 1.5 μL $^{125}IT_4$ (90000 cpm, SA 1350 μCi/μg) for 2h at room temperature and soaked in four pieces of Whatman 3MM filter paper (5 × 4 mm), were put on the cathode side of the gel. Samples were focused for 2 3 h (papers were removed after 30 min) at constant power (6W) at 8 °C up to 5500 V/h. After completion of isoelectric focusing, the gels were partially dried for 30 min *in vacuo* (at 60 °C) and exposed on AGFA XP film with an intensifying screen at 80 °C for 3 days. Thereafter, the film was developed.

Results and Discussion

A high degree of purity of the isolated TBG preparations was demonstrated by SDS-PAGE. One single band with relative molecular mass of about 55 kDa was obtained, as shown in our previous study (10).

The structure of carbohydrate moiety in isolated TBG was examined using lectins chosen for their carbohydrate specificities in accordance with the proposed composition of TBG oligosaccharides. The positions of all bands obtained in lectin blots corresponded to the molecular mass of TBG (*Figure 1*). Bands of great intensity indicated strong interactions of TBG with the following lectins: SNA I, RCA I, WGA and PHA, while the PSA band was weakly stained.

SNA I is a lectin specific for terminal sialic acid and it recognizes sequences containing sialic acid linked by α2→6 or α2→3 bonds to galactose. The positive lectin blot with SNA I (*Figure 1*, lane 1) suggests that our isolated TBG still contains sialic acid at the terminal positions. This result supports the finding of Zinn et al. (2) that NeuNAcα2→6Gal and NeuNAcα2→3Gal are present in TBG oligosaccharides, the latter occurring in oligosaccharide B. Interaction with RCA I (*Figure 1*, lane 2) originates from its binding to galactose, whether as a terminal sugar or as a part of Galβ1→4GlcNAcβ1→R residues within branched oligosaccharide chains of TBG. The positive reaction with wheat germ lectin (WGA) (*Figure 1*, lane 3) indicates the presence of either of the following sequences: Manβ1→4GlcNAcβ1→4GlcNAc and $GlcNAc\beta_1$→4GlcNAcβ1→4GlcNAc. Furthermore, this lectin possesses strong binding affinity for both N- and O-type

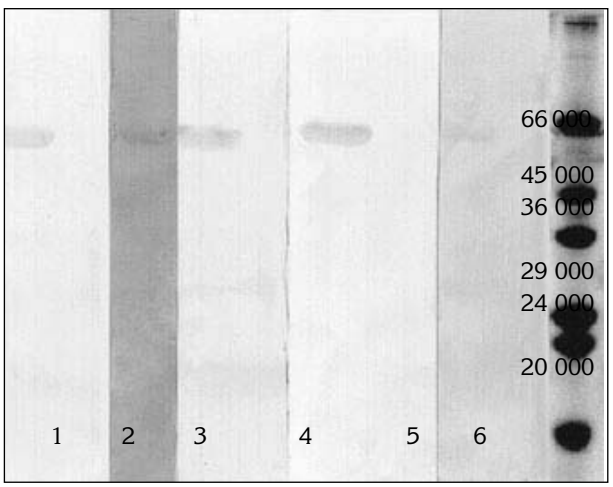

Figure 1. Lectin blot analysis of TBG. Purified TBG preparations (about 50 μg/lane, all lanes) were incubated with the following lectins: 1) SNA I, 2) RCA I, 3) WGA, 4) PHA, 5) PSA and 6) molecular weight markers with relative molecular masses indicated on the right: 66 000 bovine albumin, 45 000 ovalbumin, 36 000 glyceraldehyde-3-phosphate dehydrogenase, 29 000 carboanhydrase, 24 000 trypsinogen and 20 000 trypsin inhibitor.

oligosaccharide chains. As mentioned previously, oligosaccharides of TBG are attached via asparagine to the rest of the molecule (N-type) (1, 2). WGA also recognizes sialic acid residues but with less affinity than SNA I. Use of PHA (*Figure 1*, lane 4) provided evidence for the presence of terminal galactose as part of the Galβ1→4GlcNAcβ1→4Man sequence. This structure is present in the trianntenary oligosaccharide chain of TBG, oligosaccharide B. PSA binds strongly to oligosaccharides containing a fucose residue (Fucα1→6GlcNAc-Asn), while it binds with low affinity to Manα1→6(Manα1→3)Man. According to the chemical studies (1, 2), TBG molecules do not possess fucose residues, so the weak staining (*Figure 1*, lane 5) confirmed the presence of a trimannosyl core. Our findings are in agreement with earlier lectin affinity chromatographic studies using Con A as the most specific lectin for the trimannosyl core (6 9).

Thus, the lectin blot results showed that purified TBG reacted with lectins specific for terminal sialic acid, i.e SNA I and WGA, as well as PHA and RCA I, which detect the presence of terminal galactose residues. This indicates that our TBG preparations, besides oligosaccharide chains with intact structure (with terminal sialic acids), also contained chains terminating with galactose. The absence of some terminal sialic acid residues may have been due to partial desialylation during the isolation of TBG.

This was examined further by IEF. Namely, according to literature data (3 5, 19), TBG exhibits sialic acid dependent microheterogeneity on IEF. The results revealed a three band pattern at pI 4.2 4.6 (*Figure 2*, lanes 3 and 4) in the same position as for the reference preparation (*Figure 2*, lanes 10, 11 and 12) and sera of healthy blood donors (*Figure 2*, lanes 5, 6 and 7). Serum with very low TBG content (3.2 mg/L) showed no band after IEF (*Figure 2,* lanes 1 and 2). IEF of pregnant women sera exhibited an anodal shift (*Figure 2*, lanes 8 and 9). The appearance of one additional band of lower pI and greater anodal IEF mobility indicates more heavily sialylated TBG. Exposure to high oestrogen concentrations, as occurs in pregnant women, seems to alter the posttranslational processing of TBG resulting in the secretion of molecules of higher sialic acid content. This is responsible for the increase in the proportion of TBG bands of lower pI (20).

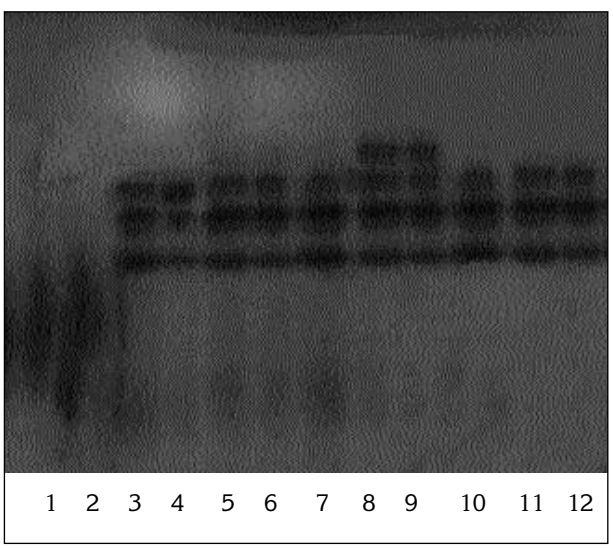

Figure 2. Isoelectric focusing of TBG on polyacrylamide gel in the pI range 4.2 4.9. Samples were incubated with $^{125}IT_4$ and the gel was autoradiographed. Lanes 1, 2 serum with very low TBG concentration (3.2 mg/L); lanes 3, 4 purified TBG preparations; lanes 5, 6, 7 sera of healthy blood donors; lanes 8, 9 pregnant women sera and lanes 10, 11, 12 reference TBG preparation. The anode is at the top of the figure.

However, some TBG preparations originating from pregnant women sera had no IEF pattern characteristic for more heavily sialylated proteins. Thus, Sviridov et al. (21) reported that the TBG variant, so-called pregnancy-associated molecular variant (TBG-1), accounted for only 10% of the total TBG in serum. The absence of the »acidic« band (with lower pI) is a result of partial removal of sialic acid from the more sialylated molecular variant of TBG. Desialylation can occur during isolation by the action of neuraminidase, which may accumulate on the affinity chromatography matrix (T_4-Sepharose 4B) after repeated reuse of the adsorbant. Another possible source of neuraminidase activity could be endogenous enzyme in plasma or bacterial contamination of the adsorbant (22).

Taken together, these studies demonstrate that purified TBG contains N-type oligosaccharide units consisting of sialic acid, galactose, N-acetylglucosamine and mannose. The proposed carbohydrate structure of TBG obtained by lectin blot analysis and IEF are in agreement with the data of Zinn et al. (2) obtained by chemical methods. Although the lectin blot results indicated the presence of desialylated TBG molecules, it seems that the removal of sialic acid had occurred in a small proportion of TBG molecules, since the IEF pattern was not altered.

In conclusion, in this study lectin blot analysis and IEF have been shown to be useful tools in the examination of TBG glycosylation.

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KARAKTERIZACIJA UGLJENOHIDRATNE KOMPONENTE PREČIŠĆENOG TBG-a LEKTINSKIM BLOTOM I IZOELEKTRIČNIM FOKUSIRANJEM

Ivana Petrović, Svetlana Savin, Ivona Baričević, Dubravka Cvejić

Institut za primenu nuklearne energije – INEP, Zemun

Kratak sadržaj: Struktura ugljenohidratnog dela prečišćenog tiroksin-vezujućeg globulina (TBG) ispitivana je lektinskim blotom i izoelektričnim fokusiranjem. U lektinskom blotu, TBG je pozitivno reagovao sa sledećim lektinima: SNA I, RCA I, WGA, PHA i PSA. Dobijeni rezultati ukazuju da prečišćeni TBG poseduje N-(glikozidno) vezane oligosaharidne lance koji se sastoje od manoze, galaktoze, N-acetilglukozamina i sijalinske kiseline. Izoelektričnim fokusiranjem dobijene su tri trake u pI opsegu od 4,2 do 4,6 čime je potvrđena očuvanost njegove ugljenohidratne strukture; nije uočena desijalinizacija TBG-a. Pokazano je da lektinski blot i izoelektrično fokusiranje mogu uspešno da se primene za izučavanje glikozilacije TBG-a.

Ključne reči: tiroksin-vezujući globulin (TBG), glikozilacija, lektini, izoelektrično fokusiranje

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