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THE ROLE OF THE CARBOHYDRATE MOIETY ON THE SIZE HETEROGENEITY AND IMMUNOLOGIC DETERMINANTS OF HUMAN TESTOSTERONE-ESTRADIOL-BINDING GLOBULIN†

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Summary—Human testosterone-estradiol-binding globulin (hTeBG)† has been purified to apparent homogeneity by several laboratories using procedures which, in most instances, were labor intensive. In this report, hTeBG was purified from pregnancy serum by a newly developed two step procedure involving sequential affinity chromatography and ion-exchange high performance liquid chromatography (ion-exchange HPLC). The purity of the final product was confirmed by silver stained SDS-polyacrylamide gel and reverse phase HPLC monitored at 206 nm. hTeBG purified by ion-exchange-HPLC maintained binding activity by Dextran coated charcoal (DCC) assay and size heterogeneity on SDS-polyacrylamide gels which were indistinguishable from those of the proteins purified by conventional chromatography. Removal of the carbohydrate moiety from the molecule by both enzymatic and chemical treatment reduced the apparent molecular size and eliminated lectin binding of hTeBG subunits. Deglycosylation did not, however, abolish or alter the distribution of the protomeric forms of this subunit. We conclude that hTeBG is a dimer whose monomer exhibits two protomeric forms which is not a result of carbohydrate heterogeneity. In addition, disialylated and deglycosylated hTeBG exhibited antigenic determinants identical to the native protein.

INTRODUCTION

Human serum contains a protein which binds androgens and estrogens called testosterone-estradiol-binding globulin (hTeBG) [1–5]. Several laboratories have purified this protein to apparent homogeneity and shown that it is a glycoprotein containing 18–34% carbohydrate [6–8]. Most of these studies have indicated that hTeBG is a dimer. In addition, recent studies form this laboratory showed that hTeBG monomers exhibit size heterogeneity on SDS-polyacrylamide gels [9]. These findings show that hTeBG is similar to the TeBG's from bull [10], dog [11], monkey [3, 12], rabbit [13], and ram (Cheng, unpublished observation) as well as the ABP's from rabbit [14], and rat [15, 16]. Even though all these dimeric proteins are composed of monomers showing distinct molecular weight variants, it was not known whether this size heterogeneity was the result of differential carbohydrate content or amino acid composition. In addition, it was not known whether the carbohydrate moiety of hTeBG influenced its binding to polyvalent antisera. These are the subjects of the present report.

EXPERIMENTAL

Materials

[1,2-^{3}H]-5α-Dihydrotestosterone ([^{3}H]-DHT, 51.6 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Radioinert DHT, bovine serum albumin (Fraction V), protein A-peroxidase, concanavalin A, wheat germ lectin (*Triticum vulgaris*) peroxidase labeled, horseradish peroxidase, 4-chloro-1-naphthol, neuraminidase (*Clostridium perfringens*), β-galactosidase (*Aspergillus niger*), and β-*N*-acetylglucosaminidase (*Aspergillus niger*) were obtained from Sigma (St Louis, MO). α-Mannosidase (*Turbo cornutus*) was from Miles Laboratories, Inc. (Elkhart, IN). Acrylamide, *N*,*N*-diallyltartardiamide (DATD), *N*,*N*′-methylene-bisacrylamide (*Bis*), ammonium persulfate, sodium dodecyl sulfate (SDS), and high molecular weight standards were from Bio-Rad Laboratories (Richmond, CA). Dextran T-70, Mono-Q™ anion-exchange HPLC column were from Pharmacia (Uppsala, Sweden). Tris (hydroxymethyl) amino-

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†The abbreviations used are: hTeBG, human testosterone-estradiol-binding globulin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ABP, androgen binding protein; DHT, 5α-dihydrotestosterone; DHT-HA-Sepharose, 3-oxo-17β-hydroxy-5α-androstane 17α-(6-hexanoic acid)-Sepharose; DCC, Dextran-coated charcoal; TG buffer, 20 mM Tris and 10% glycerol (v/v), pH 7.4 at 22°C; BSA, bovine serum albumin; TG-gelatin buffer, TG buffer containing 0.2% gelatin (w/v); %T, total gel concentration (grams per 100 ml) = acrylamide + methylene − bisacrylamide; %C, percent cross-linking =

$$100 \times \frac{\text{methylene} - \text{bisacrylamide}}{\text{acrylamide} + \text{methylene} - \text{bisacrylamide}}$$

HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid.

methane, glycine, glycerol, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), and 2-mercaptoethanol were from Bethesda Research Laboratories, Inc. (Gaithersburg, MD). Trifluoromethanesulfonic acid, pyridine, diethyl ether, anisole, and sodium chloride were from Aldrich Chemical Co., Inc. (Milwaukee, WI). Silver nitrate, methanol, hydrogen peroxide (30%, v/v) and formaldehyde solution (37%, w/v) were from Fisher Scientific Co. (Fair Lawn, NJ). Trifluoroacetic acid (Sequanal grade) was from Pierce Chemical Co. (Rockford, IL). Acetonitrile (HPLC grade) and methanol (HPLC grade) were from Burdick & Jackson Laboratories, Inc. (Muskegon, MI). Nitrocellulose paper (0.2 μm) was from Schleicher and Schuell, Inc. (Keene, NH).

General methods

[^{3}H]DHT in aqueous solution was measured by liquid scintillation spectrometry (Packard, model 3330) using 5 ml of Liquiscint (National Diagnostics, Somerville, NJ) at an efficiency of 28%. Total protein was assayed by the dye-binding method of Bradford[17] using BSA as a standard. Analytical polyacrylamide gel electrophoresis in the presence of SDS was performed as previously described [18]; the resolving gel consisted of 7.5% T and 2.6% C_{Bis} with stacking gel of 5% T and 15% C_{DATD}. Gels were stained with silver nitrate according to the procedures of Wray *et al.*[19]. The affinity resin, DHT-HA-Sepharose was prepared according to the procedures of Musto *et al.*[15, 20]. Vacuum dialysis was done on collodion bags (UH 100/10, Schleicher and Schuell, Inc.).

[^{3}H]DHT binding assay

hTeBG activity was assayed using a Dextran-coated charcoal (DCC) assay [21]. The concentration of binding sites and K_d were determined using a computer program previously described [22]. A single point DCC assay [9] was used to locate steroid binding activity in fractions eluted from the ion-exchange HPLC column. Briefly, aliquots (2 μl) from each fraction were mixed with 198 μl TG-gelatin buffer (TG buffer containing 0.2% w/v gelatin) and incubated with 2 nM [^{3}H]DHT in the absence or presence of 200-fold non-radioactive DHT to determine total and nonspecific binding, respectively. The samples were incubated for 1 h at 4°C. Bound and free steroid were separated by the addition of 0.8 ml of a Dextran-coated charcoal solution (250 mg charcoal and 25 mg Dextran T-70 in 100 ml TG buffer) which was then sedimented at 2500 ***g*** for 5 min. The radioactivity in the supernatants was assessed by liquid scintillation spectrometry.

Purification of hTeBG from pregnancy serum

Serum preparation and affinity chromatography. Blood samples obtained from pregnant women attending an outpatient clinic for routine prenatal examinations were prepared as previously described [9]. Serum (200 ml) was then fractionated on DHT-HA-Sepharose as detailed elsewhere [9]. All procedures were carried out at 4°C unless otherwise specified. The fractions containing hTeBG were concentrated to about 500 ml in an Amicon TCF-10 concentrator using a PM-10 membrane. The sample volume was further reduced to about 10 ml in an Amicon ultrafiltration cell (Model 8050) using a PM-10 membrane. The sample was dialyzed extensively against TG buffer for 24 h using a Spectrapor membrane, MW cut-off 6000–8000 from Spectrum Medical Industries, Inc. (Los Angeles, CA). It was then concentrated to about 2 ml using vacuum dialysis and filtered through a 0.22 μm filter unit (Millex-GV) from Millipore Corp. (Bedford, MA).

High performance liquid chromatography (HPLC) of hTeBG. All buffers used for HPLC were prepared with water purified from a Darco Water System (Model 344-D, Darco Water Systems, Inc. Durham, NC) and extensively degassed. HPLC was performed on a column of Mono Q™ (5 × 50 mm, i.d., strong anion exchanger) from Pharmacia using a chromatographic system from LKB (Gaithersburg, MD) consisting of a HPLC controller (Model 2152) equipped with two pumps (Model 2150) and a single wavelength u.v. detector (Uvicord SII, Model 2238). The sample injector (Model 7125), equipped with either a 2 ml sample loop, or a 200 μl loop was from Rainin Instrument Co., Inc. (Woburn, WA). All connecting tubing was 0.25 mm i.d. stainless steel except in the low pressure zone between the solvent systems and the injector where 0.75 mm i.d. tubing was used. Two ml fractions were collected using a LKB Superrac (Model 2211) fraction collector exceptor for the hTeBG peak where the peak was divided into three fractions; the ascending shoulder, the peak, and the descending shoulder. Subsequent SDS-PAGE indicated that these fractions contained purified hTeBG. The entire hTeBG peak was then collected into one tube manually in subsequent experiments. All HPLC procedures were performed at ambient temperature (22°C). Fractions containing hTeBG were transferred immediately to ice. The Tris buffer (20 mM Tris, pH 7.4 at 22°C) was prepared freshly each day and was used as the initial solvent (System A) in the gradient separations. The second solvent (System B) consisted of Tris 20 mM, NaCl 600 mM, pH 7.4 at 22°C. The normal operating pressure for Mono Q™ column was about 40 bars. In experiments where sample volume exceeded 2 ml, samples were loaded onto the column by multiple injections interspaced with a period of about 6 min during which the u.v. profile returned to baseline. Elution of proteins was performed using a linear gradient from 0 to 85% B at a flow rat of 1 ml/min in 30 min. The effluent was monitored by u.v. absorbance at 280 nm. The column was washed extensively with water and then with methanol (HPLC grade) at the end of each working day to prevent accumulation of NaCl in the tubing fittings.

For further analysis of hTeBG purity, samples (about 50 μg protein) were injected onto a Vydac C18 reverse-phase HPLC column (4.6 mm × 26 cm, i.d., particle size 5 μm) from The Separations Group (Hesperia, CA) using the 200 μl sample loop. The initial solvent (A) consisted of 25% acetonitrile–75% water and 0.1% TFA (v/v). The second solvent (B) consisted of 95% acetonitrile–5% water and 0.1% TFA (v/v). Elution was performed by a linear gradient from 0 to 40% B at a flow rate of 1 ml/min in 90 min. The effluent was monitored by u.v. absorbance at 206 nm. hTeBG collected from the reverse-phase C18 column lost all binding activity, presumably due to the low pH (pH about 2), but its presence was demonstrated by silver stained SDS-polyacrylamide gel.

Preparation of deglycosylated hTeBG

Enzymatic deglycosylation. Highly purified hTeBG was incubated with neuraminidase with and without a mixture of the following glycosidases: β-galactoside, α-mannosidase, and β-*N*-acetylglucosaminidase as previously described [9]. Briefly, hTeBG (0.1 mg protein) suspended in TG buffer was dialyzed against several changes of 0.1 M sodium acetate (pH 5.0) buffer at room temperature for 24 h and concentrated to about 0.1 ml by vacuum dialysis. About 20 μg of hTeBG was incubated in a final volume of 20 μl with the enzymes at 0.05 units/ml and incubated at 37°C for 30 h. Control tubes containing purified hTeBG, but without glycosidases, were incubated either in TG-buffer at 4°C or in 0.1 M sodium acetate buffer at 37°C. At the end of digestion, samples were dialyzed against TG buffer at room temperature for 24 h.

Chemical deglycosylation. hTeBG was deglycosylated using trifluoromethanesulfonic acid as previously described [23]. Purified hTeBG suspended in TG-buffer (about 200 μg protein) was dialyzed extensively against double-distilled water for 18 h and then freeze-dried. It was then resuspended in 90 μl of anhydrous trifluoromethanesulfonic acid and 10 μl of anisole. The reaction tube was incubated at room temperature with agitation for 6 h. At the end of the incubation, the reaction mixture was transferred to a tube containing 1 ml of pyridine–diethyl ether (1:9, v/v), in a dry ice–methanol bath. The protein and pyridinium salt of the acid co-precipitated and were collected by centrifugation at 10,000 ***g*** for 10 min. The pellet was resuspended in 0.1 M NH_4HCO_3 and dialyzed extensively against this solution. The neutralized, deglycosylated protein was then resuspended and dialyzed against 20 mM Tris buffer (pH 7.4 at 22°C) and recovered by freeze-drying.

Analysis of hTeBG by electrophoretic transfer, immuno- and lectin-staining

Native, desialylated, and deglycosylated hTeBG preparations were fractionated on SDS-polyacrylamide slab gels as described above using about 0.1 μg protein per well. Preliminary experiments indicated that this concentration of hTeBG was optimal for visualization using the immuno- and lectin-staining techniques. The protein in the slab gel was then transferred onto nitrocellulose paper using the electrophoretic transfer technique as previously described [24, 25]. After equilibrating the gel in the transfer buffer (Tris 25 mM, glycine 192 mM, pH 8.3 at 22°C containing 20% methanol, v/v) for about 10 min, the transfer of hTeBG to nitrocellulose paper was performed at 70 V (approx 0.2 Amp) for at least 12 h at room temperature. All subsequent procedures were carried out at room temperature, unless otherwise specified, with agitation on a reciprocating shaker (Eberbach Corp., Ann Arbor, MI) at 70 cpm. For immuno-staining, the nitrocellulose paper was washed twice (10 min each) with Tris–PBS buffer (20 mM Tris, 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4 at 22°C) and then incubated with 2% bovine serum albumin in Tris–PBS buffer for 3 h. It was then incubated overnight with 1% rabbit anti-hTeBG antiserum [26] in Tris–PBS buffer containing 0.1% BSA; washed twice (10 min each) in Tris–PBS buffer; and incubated with protein A-peroxidase (1 μg/ml) in Tris–PBS buffer containing 0.1% BSA. After two washings (10 min each) in Tris–PBS buffer containing 0.05% NP40 (a nonionic detergent, v/v) to remove unbound protein A-peroxidase, hTeBG bound protein A-peroxidase was visualized using 4-chloro-1-naphthol [27]. For lectin staining the procedures were essentially the same except that in place of the antiserum, lectins were used at a concentration of 10 μg/ml as detailed elsewhere [27].

hTeBG Radioimmunoassay

Purified hTeBG was iodinated by the Iodogen technique [28]. Preparation of antiserum against hTeBG and details of hTeBG radioimmunoassay procedures were performed as previously described [26]. Samples after appropriate treatment were extensively dialyzed against PBS buffer containing 0.5% BSA. Each standard or unknown sample was run in duplicate. The standard curve was calibrated against a pool of pregnancy serum with a known concentration of hTeBG [26]. Samples were diluted using 0.5% BSA in PBS. The minimal detectable dose was about 0.05 ng hTeBG per assay tube. The intra-assay coefficient of variation was 8% and inter-assay coefficient of variation was 12%. Cross reactivity of desialylated and deglycosylated hTeBG obtained by enzymatic treatment in the radioimmunoassay was determined by simultaneous fitting of displacement curves using the computer program ALLFIT [29].

RESULTS

Purification of hTeBG

The procedure for isolation of hTeBG from pregnancy serum is comprised of two chromatographic

Table 1. Summary of hTeBG purification from pregnancy serum

	hTeBG* (nmol)	Protein† (mg)	Specific activity‡ (nmol/mg)	Purification		Recovery (%)	
				From previous step	cumulative	From previous step	cumulative
Pooled pregnancy serum (200 ml)	42.4	10720	0.00396	—	1	—	100
Affinity chromatography	18.5	8.9	2.08	525	525	44	44
Ion-exchange HPLC	6.6	0.54	12.20	6	3080	36	16

*The hTeBG concentration was measured by [^{3}H]DHT binding activity using Dextran-coated charcoal assay [9].
†The protein concentration was determined by the method of Bradford [17] using bovine serum albumin as a standard.
‡Specific activity was expressed as nmol hTeBG/mg protein.

steps as outlined in Table 1. The affinity chromatography of hTeBG on DHT-HA-Sepharose produced a cumulative purification of about 500-fold over the starting material. The final HPLC on ion-exchange column produced another 6-fold increase in specific activity and increased the specific activity of hTeBG 3000-fold over that of pregnancy serum with an overall recovery of 16%. The retention time for hTeBG was approx 22 min, therefore its exposure to ambient temperature was less than 25 min (Fig. 1). Fractions containing specific [^{3}H]DHT binding activity corresponded to the first major protein peak (peak 1, Fig. 1) and eluting at approx 180 mM NaCl. The second major peak (peak 2, Fig. 1) showed minor [^{3}H]DHT binding activity. Silver stained SDS-polyacrylamide gels indicated this protein to be albumin (data not shown).

Peak 1, which contained most of the binding activity, exhibited only two protein bands on SDS gels (lane 2, Fig. 2) which were identical to the heavy (H) and light (L) protomers of hTeBG ($hTeBG_H$ and $hTeBG_L$) described previously [9]. To further assess protein purity, a sample from peak 1 was fractionated on a reverse-phase C18 column. A single protein peak was eluted from this column (Fig. 3). The preparation purified by anion-exchange HPLC was shown to possess binding activity with an apparent K_d at 4°C of about 1.2×10^{-9} M and a specific binding activity

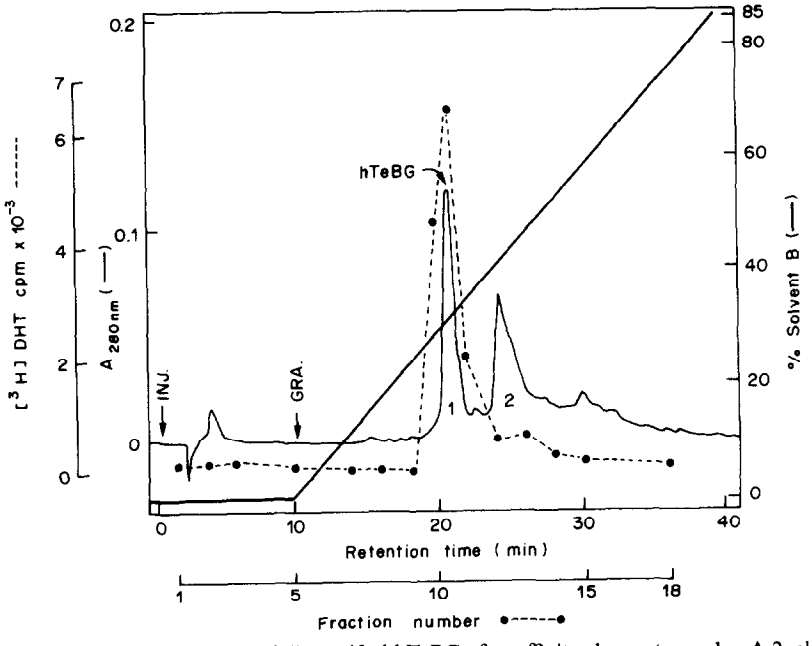

Fig. 1. Anion-exchange HPLC of partially purified hTeBG after affinity chromatography. A 2 ml sample was dialyzed extensively against solvent system A and injected into a Mono Q™ column. After injection, solvent A (20 mM Tris, pH 7.4 at 22°C) was passed through the column for 10 min. A gradient of 0–85% solvent B (20 mM Tris, 600 mM NaCl, pH 7.4 at 22°C) was then applied over 30 min, then 100% B for 5 min at a flow rate of 1 ml/min. Two ml fractions were collected except for the hTeBG peak which was collected in three fractions manually. An aliquot (2 μl) from each fraction was mixed with 198 μl TG-gelatin buffer and incubated with 2 nM [^{3}H]DHT in the absence or presence of 200-fold excess radioinert DHT at 4°C for 1 h to estimate total and non-specific binding, respectively. Unbound [^{3}H]DHT was removed by DCC. All samples were assayed in duplicate. Specific binding was estimated (●——●) by subtracting non-specific from total binding. INJ indicates sample injection; GRA indicates beginning of gradient.

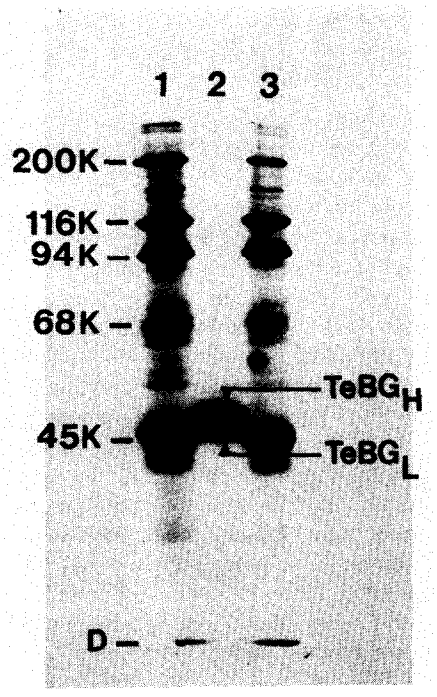

Fig. 2. Silver stained SDS-polyacrylamide gel of purified hTeBG after ion-exchange HPLC. Lanes 1, 3 are molecular weight standards consisting 0.8 μg each of the following proteins: Myosin, 200,000; β-galactosidase, 116,000; phosphorylase b, 94,000; bovine serum albumin, 68,000; and ovalbumin, 45,000. Lane 2 is purified hTeBG (about 1 μg protein). The H and L indicate the relative mobilities of hTeBG$_H$ and hTeBG$_L$. K = X1000, D—dye front.

of 12.2 nmol/mg protein, comparable to those obtained by conventional chromatography [9]. The analysis of the final preparation by specific activity, SDS–PAGE, and reverse-phase HPLC suggested that the protein in peak I was homogeneous hTeBG. In addition, two different batches of hTeBG purified from pregnancy serum were indistinguishable.

Deglycosylation of hTeBG

To determine whether removal of the sialic acid or carbohydrate moieties of hTeBG would affect the distribution of its protomers, purified hTeBG was deglycosylated by both enzymatic and chemical procedures, resolved on SDS containing polyacrylamide gels and transferred electrophoretically onto nitrocellulose paper. The location of the protein was visualized by immuno-staining. The distribution of H and L protomers in the native protein by this technique (Figs 4 and 5A) was essentially the same as shown by silver staining (Fig. 2). The protomers from the desialylated and deglycosylated preparations of hTeBG appear to migrate faster on SDS-polyacrylamide gels and correspond to an apparent change in mol. wt of about 3000–4000 (Figs 4, 5A). In addition the relative amounts of the two protomers following removal of carbohydrate appeared to be the same as in the untreated protein.

The effect of desialylation and deglycosylation treatments on lectin binding was also assessed. hTeBG treated with neuraminidase showed the expected shift in size on SDS-polyacrylamide gel but retained binding to concanavalin A (Fig. 4, lane 8) which is specific for α-D-mannose, α-D-glucose and sterically related residues [30] while deglycosylated hTeBG showed no binding to concanavalin A (Fig. 4, lane 5). Chemically deglycosylated hTeBG showed a similar shift in apparent size on all SDS containing polyacrylamide gels but lost binding to concanavalin A and wheat germ agglutinin (Fig. 5B). This latter lectin is specific for *N*-acetyl-glucosaminyl and sialic acid residues [30].

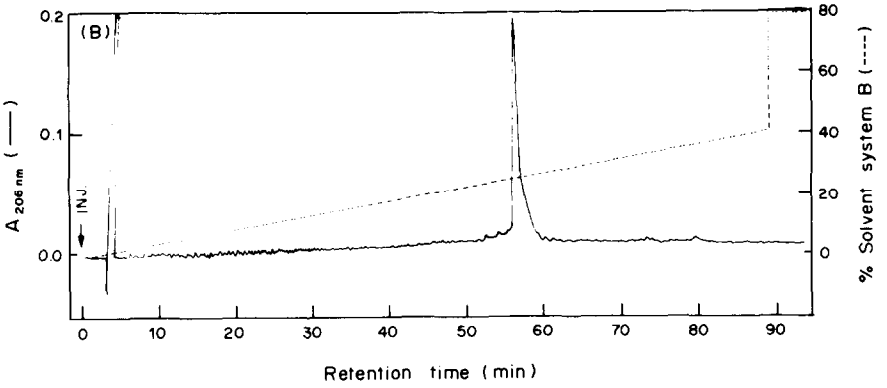

Fig. 3. Reverse-phase HPLC of purified hTeBG. Purified hTeBG (about 50 μg protein in 100 μl TG buffer) was injected onto a Vydac C18 reverse-phase column (4.6 mm × 25 cm, 5 μm particle size) equilibrated with solvent A [25% acetonitrile–75% water and 0.1% TFA (v/v)]. A 0–40% gradient of solvent B [95% acetonitrile–5% water and 0.1% TFA (v/v)] was started at the time of sample injection. The eluate from the column were monitored by u.v. absorbance at 206 nm. Flow rate was 1 ml/min. For re-equilibration, solvent system A was passed through the column at a flow rate of 1 ml/min for 15 min. Only one major protein peak was identified. When this peak was concentrated and fractionated on a SDS containing polyacrylamide gel, only two bands with the apparent molecular weights of the H and L protomers of TeBG were detected with silver stain.

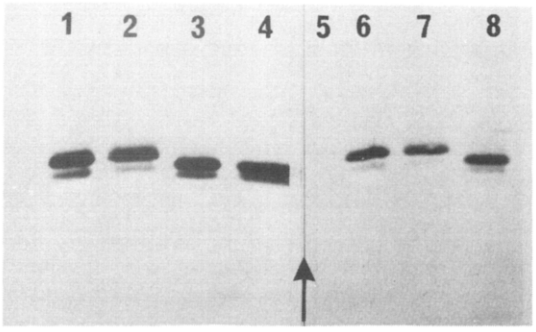

Fig. 4. Relative mobilities of intact, enzymatically desialylated, and enzymatically deglycosylated hTeBG. These proteins (about 0.1 μg purified hTeBG per lane) were fractionated on a single 7.5% SDS-polyacrylamide gel and transferred electrophoretically to nitrocellulose paper. The nitrocellulose paper was divided into two equal portions as indicated by the arrow and the TeBGs were visualized as described in Materials and Methods by either immunological staining (lanes 1–4) or lectin (5–8) staining using concanavalin A. Lane 1, purified hTeBG incubated in TG buffer at 4°C for 30 h without glycosidases; lane 2, purified hTeBG incubated in 0.1 M sodium acetate buffer (pH 5.0) at 37°C for 30 h without glycosidases; lane 3, desialylated hTeBG; lane 4, deglycosylated hTeBG; lane 5, deglycosylated hTeBG. lane 6, hTeBG incubated in TG buffer at 4°C for 30 h without glycosidases; lane 7, hTeBG incubated in 0.1 M sodium acetate buffer (pH 5.0) at 37°C for 30 h without glycosidases; lane 8, desialylated hTeBG. Electrophoresis was from top to bottom.

Cross-immunoreactivity of desialylated and deglycosylated hTeBG with antiserum raised against purified native hTeBG

To determine whether the removal of sialic acid and carbohydrate moieties from purified hTeBG would affect its antigenic determinants, enzymatically treated and intact samples of this protein were dialyzed extensively against PBS buffer containing 0.5% BSA. Each hTeBG sample was then used to generate displacement curves in the hTeBG radioimmunoassay (Fig. 6). In two separate experiments, intact, desialylated and deglycosylated hTeBG produced complete displacement of [^{125}I]hTeBG from the antibody. Analysis of these data by simultaneous fitting to four parameter logistic curves using ALLFIT [29] indicated regardless of treatment, complete and parallel displacements for all hTeBG preparations were obtained. These results show that both desialylated and deglycosylated hTeBG have complete cross reactivity with antiserum to native hTeBG and suggest that the carbohydrate moieties of this protein are not important epitopes.

DISCUSSION

We have described a procedure where highly purified hTeBG can be obtained from pregnancy

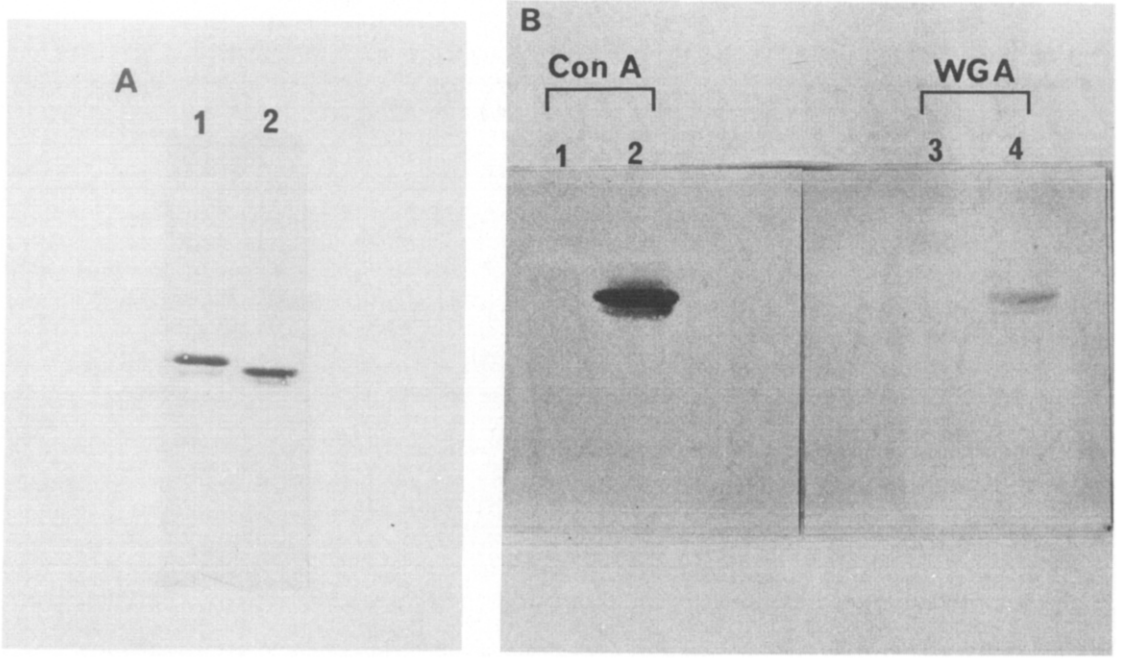

Fig. 5. Relative mobilities of intact and chemically deglycosylated hTeBG. These proteins (about 0.1 μg purified hTeBG per lane) were fractionated on SDS-polyacrylamide gels, transferred to nitrocellulose paper, and then stained using either anti-hTeBG (panel A) or lectins (panel B) as described in the Materials and Methods. (A): Lane 1, intact hTeBG; lane 2, chemically deglycosylated hTeBG. B: Lanes 1 and 3 were deglycosylated hTeBG; lanes 2 and 4 were intact hTeBG. Electrophoresis was from top to bottom. Con A, concanavalin A; WGA, wheat germ agglutinin.

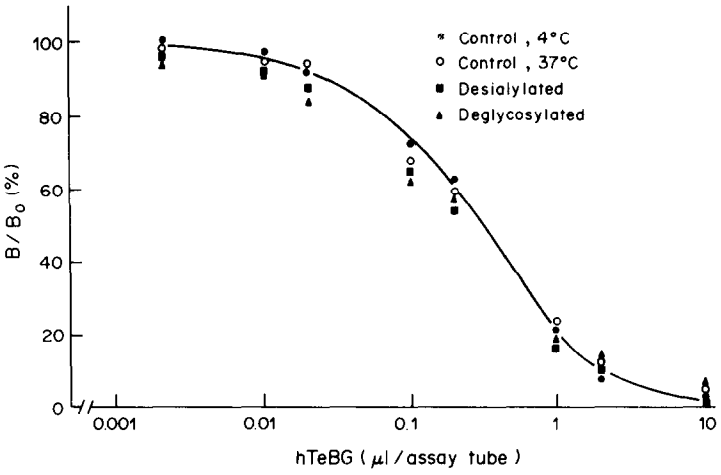

Fig. 6. Competition of [^{125}I]hTeBG with intact, enzymatically desialylated, and enzymatically deglycosylated hTeBG for binding to hTeBG antiserum. The abscissa is the log dose of competitor. The ordinate is expressed as B/Bo, where B and Bo are counts bound in the presence and absence of unlabeled competitor, respectively.

serum within five working days using affinity chromatography and ion-exchange HPLC. The main advantages of ion-exchange HPLC are speed and reproducibility. Preliminary results suggest this technique can also be used to purify TeBG from the bull, rabbit and ram as well as ABP from the rabbit, ram and man (Cheng, unpublished observations). The lower yield obtained with this procedure as compared to previous purifications (16 vs 27%) [9] was due to lower recovery from the affinity chromatography step rather than from HPLC. It should be noted that the same batch of affinity resin was used in both this and previous studies. It gave only a 40% recovery in this study compared with 60% in our previous report [9]. But the recovery of hTeBG by HPLC over that of the partially purified material from affinity chromatography was about 40%, comparable to the hydroxylapatite chromatography.

Purified hTeBG obtained by this procedure showed size heterogeneity as previously described [9]. Since the size heterogeneity of a glycoprotein could be the result of its carbohydrate content [31, 32, 33] we thought it pertinent to determine whether this was the case for hTeBG. When a mixture of exoglycosidases was used to remove the carbohydrate moiety from hTeBG, both the size heterogeneity and the distribution of hTeBG monomers between $hTeBG_H$ and $hTeBG_L$ was maintained even though the size of the protomers decreased relative to intact monomers.

To confirm the results of enzyme treatment, a technique for chemical deglycosylation was used which employed trifluoromethanesulfonic acid [23]. This reagent cleaves all *O*-glycosyl bonds and *N*-glycosyl bonds except the *N*-glycosyl bond between *N*-acetylglucosamine and asparagine. Recently it has been shown that each hTeBG molecule contains two biantennary *N*-linked oligosaccharide chains of the *N*-acetyllactosamine type and one *O*-linked oligosaccharide chain [34]. We have therefore used trifluoromethanesulfonic acid to cleave the carbohydrate chains from hTeBG. The effectiveness of this treatment has been demonstrated by different lectin binding techniques. It was shown that chemically deglycosylated hTeBG maintained size heterogeneity similar to the untreated protein.

The fact that chemically deglycosylated hTeBG, which presumably retained the *N*-acetylglucosamine attached to the asparagine [23], lost lectin binding for wheat germ agglutinin, which is specific for *N*-acetylglucosaminyl residues, suggests that either the number of *N*-acetylglucosamine residues left was too small to permit visualization at the protein concentrations used or its steric configuration did not permit binding to the wheat germ agglutinin. The ability of wheat germ agglutinin and concanavalin A binding to visualize untreated hTeBG but not chemically deglycosylated implies that most of the carbohydrate moiety had been removed (Fig. 5B).

The inability of either enzymatic or chemical deglycosylation to change the relative distribution of H and L protomers in hTeBG suggests the differences in $hTeBG_H$ and $hTeBG_L$ are in their amino acid sequence or composition rather than carbohydrate moieties. This conclusion is consistent with a previous study which indicated that $hTeBG_H$ had one more polypeptide than $hTeBG_L$ after digestion with *Staphylococcus aureus* V_8 [9]. Proof of this assumption must await sequence analysis of both H and L protomers.

In summary, a rapid purification procedure for hTeBG involving ion-exchange HPLC has been described. Studies on highly purified hTeBG suggests that the size heterogeneity of the protomers is not the result of carbohydrate heterogeneity and that the carbohydrate moiety is not an important antigenic determinant on hTeBG.

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