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Influence of glycosylation on the clearance of recombinant human sex hormone-binding globulin from rabbit blood

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Abstract

Human sex hormone-binding globulin (hSHBG) is a plasma glycoprotein that binds sex steroids with high affinity. Variations in hSHBG glycosylation contribute to its electrophoretic microheterogeneity, but the functional significance of different SHBG glycoforms is unknown. Carbohydrates may influence the biological activities and half-lives of glycoproteins and we have examined how oligosaccharides at specific sites influence the plasma clearance of hSHBG in vivo. To accomplish this, fully-glycosylated hSHBG, or hSHBG mutants lacking specific oligosaccharides chains, were expressed in Chinese hamster ovary (CHO) cells and purified by immunoaffinity chromatography. The purified recombinant proteins were then biotinylated to study their plasma half-lives after intravenous injection into rabbits. When compared to hSHBG isolated from serum, recombinant hSHBG migrates with a slightly larger average molecular size during denaturing polyacrylamide gel electrophoresis. This is due to a greater proportion (33–39% vs. 3%) of more highly branched *N*-linked oligosaccharides on the recombinant proteins. When injected into rabbits, the disappearance of recombinant hSHBG showed two exponential components, as previously shown for natural hSHBG in the same animal model. The mean ± S.E.M. plasma half-lives of recombinant hSHBG ($t_{1/2}\alpha$ 0.11 ± 0.03 h and $t_{1/2}\beta$ 18.94 ± 1.65 h) are shorter than previously measured for natural hSHBG ($t_{1/2}\alpha$ 3.43 ± 0.72 h and $t_{1/2}\beta$ 38.18 ± 7.22 h) and this is likely due to differences in the composition of their *N*-linked oligosaccharides. An *O*-linked chain at Thr^7 does not influence the plasma clearance of hSHBG in the presence or absence of *N*-linked carbohydrates at Asn^{351} and Asn^{367}. However, a 1.5–1.6 fold ($p < 0.03$) increase in plasma half-life of variants lacking both *N*-glycosylation sites was observed and this is probably due to the fact these variants are not recognized by the asialoglycoprotein receptor-mediated clearance system. Removal of either *N*-glycosylation consensus site also increased ($p < 0.0001$) the plasma half-life of hSHBG by 2.3–2.4 fold. Thus, the metabolic clearance of hSHBG appears to be determined by the number of *N*-linked oligosaccharides rather than their location.

1. Introduction

Sex hormone-binding globulin (SHBG) is a transport protein for sex steroid hormones in the blood of humans and many other mammals [1–4]. Human SHBG (hSHBG) is a homodimeric glycoprotein with a single high affinity binding site for testosterone and estradiol [5]. It has an *O*-glycosylation site at Thr^7 and *N*-glycosylation sites at Asn^{351} and Asn^{367} [6] which are occupied almost exclusively by biantennary carbohydrate chains of the *N*-acetyllactosamine type [7]. Although these oligosaccharides do not influence SHBG subunit association or steroid-binding activity [8,9], a consensus site for *N*-glycosylation is present in an analogous position relative to the carboxy-termini

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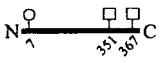
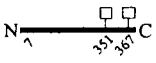
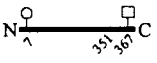
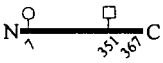
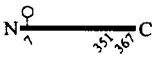
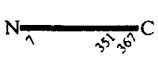
Table 1
Half-lives values ($t_{1/2}\alpha$ and $t_{1/2}\beta$), expressed in hours, measured for the different biotin labeled hSHBG glycoforms purified from conditioned CHO cells culture medium (M ± S.E.M. of 3 experiments). The six different recombinant hSHBG produced by transfected CHO cells are shown schematically as amino-acid backbones (black line), with their corresponding *O*- (○) or *N*-glycans (□). Amino (N)- and carboxy (C)-terminal extremities are indicated. Numbers refer to the amino acids residues where oligosaccharide attachment sites are located in the hSHBG amino-acid sequence

	SHBG	SHBG-O^{7-}	SHBG-N^{351-}	SHBG-N^{367-}	SHBG-$N^{351,367-}$	SHBG-O^{7-},$N^{351,367-}$
						
$t_{1/2}\alpha$	0.11±0.03	0.40±0.11	5.19±1.46 *	3.21±0.61 *	3.55±0.21 *	3.86±0.78 *
$t_{1/2}\beta$	18.94±1.65‡	19.46±2.35‡	42.51±3.52‡*	45.28±0.97‡*	27.95±3.51 *	29.57±1.79 *

* Statistical differences from SHBG.
‡ Statistical differences from SHBG-O^{7-},$N^{351,367-}$.

of all mammalian SHBG sequences identified to date (Asn^{367} in hSHBG) and this suggests that it is functionally important [10,11].

Carbohydrate chains influence the half-life of many glycoproteins in the blood [12–16] and we have shown previously that the product of a variant human s*hbg* allele, which contains an additional *N*-glycosylation site at Asn^{327}, has a prolonged half-life in rabbits when compared to normal hSHBG [17]. This raised the possibility that *N*-linked oligosaccharides influence SHBG metabolism and that SHBG clearance from the blood is determined by either the number or location of oligosaccharides attached to its polypeptide backbone.

To address this question, we have produced wild-type hSHBG and various hSHBG mutants lacking specific glycosylation sites in Chinese hamster ovary cells [8]. These recombinant proteins were then purified by immuno-affinity chromatography and labeled with biotin to trace their plasma half-lives after intravenous administration into rabbits [17,18]. The use of biotinylation as a labelling technique for these types of studies offers several advantages over radio-labeling with Iodine-125 and its high sensitivity is well suited to experimental protocols that rely on the use of relatively small amounts of recombinant proteins obtained from mammalian cells in culture [18]. During the course of these studies, it was noted that the plasma half-life of hSHBG produced by CHO cells differed from that of SHBG purified from human serum [17] and we have attributed this to qualitative differences in their associated carbohydrates chains. Despite these differences, our experimental protocol has allowed us to demonstrate that the plasma half-life of hSHBG is determined by the number rather than the location of *N*-linked oligosaccharides associated with it.

2. Materials and methods

2.1. Animals

Male New Zealand rabbits (Elevage Scientifique des Dombes, Chatillon sur Chalaronne, France) were housed in individual cages. They weighed 3065 ± 424 g and were fed with standard commercial rabbit chow and water ad libitum.

2.2. Reagents

Cell culture media and reagents and Nunc Doubletray units were purchased from Life Technologies (Grand Island, NY). Other culture flasks were obtained from Falcon Plastics (Oxnard, CA). Biotin *N*-hydroxysuccinimide ester (BIOTIN-X-NHS) was from Calbiochem® (Meudon, France), and an ECL-detection kit and the Hybond™-ECL nitrocellulose membranes were from Amersham (Les Ulis, France). Neuraminidase (EC 3.2.1.18) and methyl α-D-mannopyranoside (mannoside) were from Sigma (St. Louis, MO) and other routine chemicals were from Merck (Darmstadt, Germany).

2.3. Cell culture and transfection

Expression vectors containing cDNAs for wild-type hSHBG or hSHBG mutants lacking specific carbohydrate chains (Shown schematicaly in Table 1) were constructed and transfected into Chinese hamster ovary (CHO, pro$^-$ wild type) cells, as described previously [8]. Transfected CHO cells were cloned by limiting dilution and clones producing the highest concentrations of SHBG, as measured by a specific immunoradiometric assay (IRMA) for hSHBG (^{125}I-SBP-Coatria, bioMérieux SA, Marcy l'Etoile, France), were selected for expansion in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine (30 g/l), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). When the cells reached confluence in Doubletray units at 37°C and 5% CO_2, the medium was harvested every 3–4 days, until at least 2 l of medium was obtained and stored at −20°C until used for SHBG purification.

2.4. Electrophoresis and western blotting

The SDS-PAGE and western blots of recombinant hSHBG were performed essentially as described previously for serum SHBG [17]. In brief, medium samples were diluted to a final SHBG concentration of 5 nM in phosphate-buffered saline and SHBG was then semi-purified from 1 ml samples using a monoclonal antibody coupled to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology). The immunoadsorbed SHBG was removed from the antibody-coupled Sepharose by boiling in SDS-PAGE loading buffer and was subjected to SDS-PAGE with 4% and 7.5% acrylamide stacking and resolving gels, respectively. Proteins were transferred electrophoretically from the gel onto a nitrocellulose membrane. The membrane was incubated with a rabbit-derived anti-hSHBG polyclonal antisera and SHBG was detected by a horseradish peroxydase-labeled, secondary antibody detection system (ECL-detection kit) according to the manufacturer's protocol.

2.5. Purification and biotin labeling of hSHBG

Wild-type and mutant forms of recombinant hSHBG were isolated from the CHO cell culture media by immuno-affinity chromatography using an immobilized monoclonal anti-hSHBG antibody [19]. The purity of hSHBG isolated in this way was checked by SDS-PAGE (10% acrylamide resolving gel) followed by silver staining of the electrophoresis gel [20].

Purified SHBG (2 nmol) diluted in 200 μl of Tris buffer saline (TBS, 50 mM Tris-HCl, pH 7.4, NaCl 0.14 M) was incubated with 5 μl of a 44 mM biotin-X-NHS solution in dimethylformamide (DMF) for 2 h at 4°C and excess labeling reagent was removed by dialysis against 1 l of TBS overnight at 4°C [18].

2.6. Analyses of hSHBG glycoforms by concanavalin-A (Con-A) chromatography and desialylation

Samples containing hSHBG were subjected to Con-A chromatography to assess the types of carbohydrate chains (bi-antennary vs. more branched oligosaccharides) associated with each hSHBG glycoform [8]. In brief, a 5 ml column of Concanavalin A-Sepharose (Pharmacia LKB Biotechnology) was pre-equilibrated in Con-A buffer (50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$). Serum or culture medium containing hSHBG was applied on the column, which was washed with Con-A buffer until non-bound proteins were removed. The Con-A buffer containing 20 mM mannoside and 5% DMF was then applied and eluted proteins were collected in 1 ml fractions. An IRMA was used to identify fractions containing Con-A retarded and non-retarded forms of hSHBG. These fractions were pooled separately and analysed by western blotting, as described above.

To demonstrate the presence of sialic acid residues, culture media containing 5 pmol SHBG were first diluted in 25 mM acetate buffer, pH 5.8, to a final volume of 2 ml. To this, 1 μl 5 N HCl and 10 μl neuraminidase (4 U/ml) were added and incubated for 5 h at 37°C. The neuraminidase-treated or untreated samples were then analyzed by western blotting, as described above.

2.7. Determination of hSHBG half-life in rabbits

Biotin-labeled hSHBG glycoforms were injected into a marginal ear vein of rabbits (n = 3 for each glycoform). Blood (0.5 to 2 ml) was drawn from the contralateral ear vein at 2, 10, 20, 30, 40, 50 min and at 1, 2, 4, 8, 12 h and then every 12 h for 4 days. Serum was prepared and stored at −20°C until analysis.

The biotin-labeled hSHBG in rabbit sera was immobilized using tubes coated with monoclonal anti-hSHBG mouse antibody [17,18] and then detected in a luminometer operating in the photon counting mode by using alkaline phosphatase conjugated-streptavidin (AVIDx-AP™), together with an alkaline phosphatase substrate (AMPPD®) and the EMERALD™ green chemiluminescence amplifier (Tropix, Bedford, MA), as described previously [18].

2.8. Statistical analysis

The amounts of recombinant biotinylated hSHBG in rabbit serum samples were plotted against time and the resulting curves were analysed by regression analy-

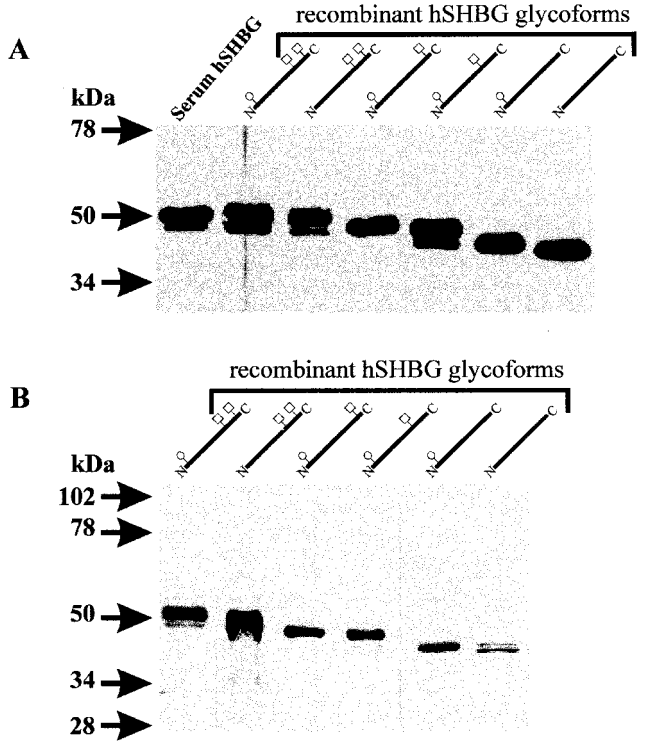


Fig. 1. (A) Comparison of the electrophoretic properties of natural hSHBG and recombinant wild-type hSHBG and hSHBG glycosylation mutants produced by CHO cells. Immuno-affinity purified hSHBG from serum and CHO cell medium was analyzed by SDS-PAGE and western blotting. (B) Purity of immuno-affinity purified recombinant hSHBG as assessed by SDS-PAGE and silver staining. Molecular size markers are on the left.

sis using SigmaPlot® software (Jandel Scientific, Erkrath, Germany). The signal disappearance best fitted a two-exponential decrease and the half-life for each component was calculated.

One-way analysis of variance was used to determine the significance of differences between half-life mean values. Individual comparisons between pairs were performed by Fisher's protected least significant procedure. $p < 0.05$ was considered significant. Results are presented as mean $\pm$ SEM.

3. Results

3.1. Electrophoretic characteristics and purity of hSHBG glycoforms used for half-life studies

The different forms of human SHBG produced by transfected CHO cells display differences in their relative mobility during SDS-PAGE and fully glycosylated recombinant SHBG migrates with a slightly larger Mr than SHBG from human serum (Fig. 1A). Removal of each glycosylation site enhanced the mobility of the protein and elimination of both *N*-glycosylation site suppressed the size heterogeneity normally associated

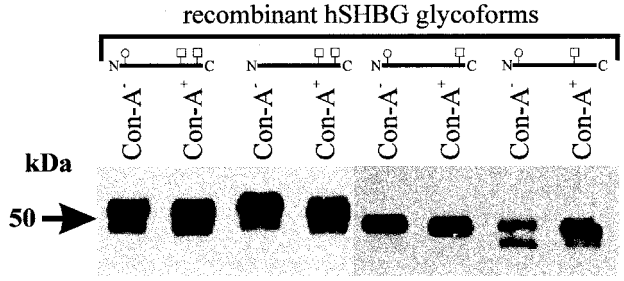


Fig. 2. Western blot of recombinant wild-type hSHBG and hSHBG glycosylation mutants separated by lectin-affinity chromatography. The recombinant hSHBG glycoforms in CHO cell medium were fractionated by chromatography on a Concanavalin-A (Con-A) Sepharose column. Excluded (Con-A$^-$) and mannoside-eluted (Con-A$^+$) fractions were pooled separately and the hSHBG was immuno-affinity purified for analysis by SDS-PAGE and western blotting. The position of a molecular size marker is shown on the left.

with hSHBG (Fig. 1A). The purity of the various hSHBG glycoforms used for half-life studies was assessed by silver staining of an analytical SDS-PAGE gel (Fig. 1B) and was 87–99%. This level of purity was acceptable for plasma half-life studies because the detection of biotinylated hSHBG in blood samples was based on a highly specific immuno-chemical detection system (see above).

3.2. Carbohydrate composition of SHBG glycoforms

Since removal of *N*-linked carbohydrates chains abolishes the Con-A-binding properties of SHBG [8], we did not subject unglycosylated hSHBG and hSHBG containing only an *O*-linked carbohydrate chain, to Con-A chromatography. Only a very small percentage (3%) of SHBG purified from human serum fails to bind Con-A Sepharose during chromatography [8], while a much greater proportion of wild-type recombinant hSHBG (39%) and the hSHBG mutants lacking either *N*-linked carbohydrate chain (33–39%) is not retarded by the Con-A Sepharose chromatography column (data not shown). However, Con-A chromatography of various recombinant hSHBG glycosylation mutants indicated that the Con-A-retarded forms, which constituted the major fraction, could be completely displaced from the lectin under exactly the same conditions (i.e. 20 mM mannoside) as the Con-A-retarded fraction of hSHBG in blood samples (data not shown) and this suggests they are very similar in terms of their carbohydrate composition.

When the lectin-excluded (Con-A$^-$) and lectin-retarded (Con-A$^+$) glycoforms of the wild-type hSHBG and the glycosylation mutants where subjected to SDS-PAGE and western blotting, the Con-A$^-$ glycoforms all migrated with a slightly lower relative electrophoretic mobility than the Con-A$^+$ glycoforms (Fig. 2) and this is consistent with the association of

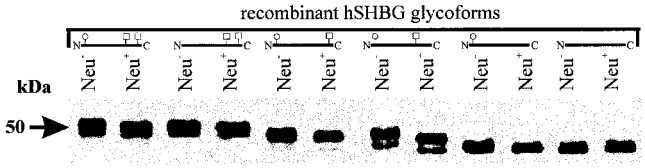


Fig. 3. Western blot of recombinant wild-type hSHBG and hSHBG glycosylation mutants before and after desialylation with neuraminidase. Samples of SHBG (5 pmol) were desialylated with 0.04 U of neuraminidase. The hSHBG in reaction mixtures was immuno-affinity purified for analysis by SDS-PAGE and western blotting. The position of a molecular size marker is shown on the left.

more highly branched, carbohydrate chains with Con-A$^-$ glycoforms. The relative proportions of glycoforms with more highly branched oligosaccharides was similar in all but one of the recombinant forms of hSHBG, namely the mutant lacking a glycosylation at residue-367, in which case the relative amount of highly-branched glycoform was much reduced and almost equal to the amount of unglycosylated protein.

Wild-type CHO cells lack the β-galactoside α 2,6-sialyltransferase that may be responsible for the NeuAcα2,6Gal linkage normally found in the *N*-carbohydrate chains associated with SHBG [7,21]. However, they have the capacity to sialylate proteins with *N*-linked oligosaccharides terminating in a NeuAcα2,3Gal sequence [21]. To assess whether the carbohydrate chains on the various hSHBG glycoforms produced by CHO cells were at least partially sialylated, we compared their electrophoretic mobility by western blot analysis, before and after neuraminidase treatment. Consistent with the removal of sialic acid residues, desialylation enhanced the electrophoretic mobility of all recombinant forms of hSHBG that contain at least one of the three possible glycosylation sites, whereas there was no change in the mobility of unglycosylated hSHBG (Fig. 3).

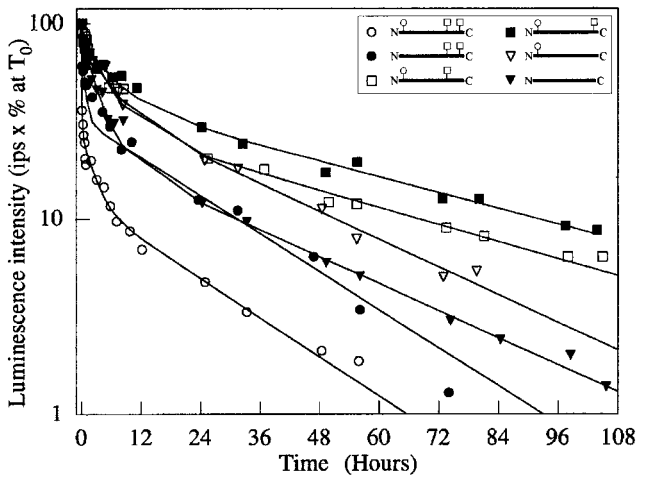


Fig. 4. Half-life of biotinylated recombinant wild-type hSHBG and hSHBG glycosylation mutants in rabbit blood. Results are indicated as percentage of the signal value (ips = impulses per second) at time zero (T_0). For each protein, representative data points from one of three identical experiments is shown.

3.3. Plasma half-life of hSHBG glycosylation mutants in rabbits

The disappearance of all forms of biotinylated recombinant hSHBG from rabbit blood was resolved into two exponential components (Fig. 4). From each component, a plasma half-life was calculated and referred to as the $t_{1/2}\alpha$ and $t_{1/2}\beta$ and the plasma half-life measurements for each SHBG glycosylation mutant are compared to values obtained using wild-type recombinant hSHBG in Table 1. These data clearly demonstrate that removal of the *O*-glycosylation site from wild-type hSHBG has no influence on plasma half-life and its presence alone also has no effect when compared to unglycosylated hSHBG. Removal of either one or both *N*-glycosylation sites resulted in a significant increase in the $t_{1/2}\alpha$ and $t_{1/2}\beta$ of these hSHBG mutants, when compared to the corresponding half-life measurements for the wild-type control. However, removal of both *N*-linked chains had much less of an impact than just removing either one of them alone.

4. Discussion

The plasma half-lives of SHBG isolated from human and rabbit blood are very similar when measured in rabbits [17,18] and this probably reflects their high degree of polypeptide sequence identity, as well as a perfect conservation in the positioning of their *N*-glycosylation sites [6,22]. However, when compared to hSHBG isolated from serum, the protein produced by CHO cells has a significantly shorter biological half-life in rabbits. We attribute this primarily to differences in their carbohydrate composition because the glycosylation of hSHBG is cell-type specific [23] and may vary with culture conditions [24]. This can be most easily appreciated by using lectins, such as Concanavalin A, to separate different glycoforms and only about 60% of hSHBG glycoforms produced by CHO cells interact with Concanavalin-A, while almost all of hSHBG in serum interact with this lectin, as reported previously [23]. The recombinant hSHBG glycoforms that fail to interact with Con-A largely comprise more branched oligosaccharides, as evidenced by their lower mobility during SDS-PAGE. The glycoforms that do interact with Con-A, however, appear to bind it with similar affinity as natural hSHBG glycoforms and this suggests that they are very similar in terms of their carbohydrate compo-

sition [25]. Although we cannot be sure what impact the presence of more highly branched carbohydrates has on the half-life of the protein, it is probably not very significant because hSHBG mutants with only one *N*-glycosylation site have very similar half-lives, despite the fact that they have very different proportions of bi-antennary and more branched chains associated with them.

For glycoproteins that comprise *N*-linked oligosaccharides, desialylation enhances their uptake by the hepatic asialoglycoprotein receptor (AGP-R) which binds oligosaccharide moieties with a terminal galactose [26]. This explains why desialylation markedly enhances the uptake of asialo-hSHBG by the rat liver [27] and why asialo-rabbit SHBG is cleared extremely rapidly ($t_{1/2}$20 min) from rabbit plasma [18]. Although the CHO cells used to express hSHBG have all the necessary glycosyl-transferases for addition of complex oligosaccharides of the *N*-acetyllactosamine type, they do not express the β galactoside α 2,6-sialyltransferase [21]. Therefore, they may not add sialic acid residues in the same way as hepatocytes and the recombinant proteins they produce may be incompletely or abnormally sialylated. Together with the fact that partially desialylation of recombinant hSHBG may occur during prolonged exposure to cells in culture, this is probably the main reason for the difference in its plasma half-life when compared to natural hSHBG isolated from serum. Despite this, we have been able to compare the behavior of various hSHBG glycosylation mutants in the rabbit model and have gained insight into how particular carbohydrate chains influence the half-life of this plasma glycoprotein.

It is not known how much SHBG is produced without *N*-linked carbohydrates by the human liver, but our data indicate that unglycosylated hSHBG or hSHBG with only a single *O*-linked carbohydrate chain are both cleared relatively rapidly from the blood. Removal of unglycosylated hSHBG from the blood circulation cannot involve the AGP-R and may simply be effected by renal filtration. Although this process has been reported to be inhibited by both size and surface charge increase due to bound oligosaccharides [28], it does not appear to be influenced by the *O*-linked oligosaccharide at Thr^7. In fact, the *O*-linked chain seems to have essentially no influence on SHBG clearance and this is consistent with the concept that the clearance of fully-glycosylated hSHBG is mediated primarily by interaction of *N*-linked carbohydrates with the AGP-R [26,27].

Human SHBG circulates in the blood as a mixture of different isoforms [5,29] which are the result of partial utilization of the two *N*-glycosylation sites on each SHBG subunit [8]. Transfected CHO cells also produce a complex mixture of different SHBG isoforms, which can be variably glycosylated and the structural complexity of their bound carbohydrates can differ. One of our major goals was therefore to determine whether oligosaccharides at these two sites influence the plasma clearance of the protein in distinct ways and our results clearly indicate that their position does not affect this process. Thus, it remains to be determined why a consensus site for *N*-glycosylation has been so efficiently conserved at a specific position close to the carboxy-terminus of SHBG throughout mammalian evolution. One possibility is that an oligosaccharide at this location provides some other function that might be related to the extra-vascular compartmentalization of SHBG [30] and/or its ability to interact with cell surface binding sites which display a high degree of specificity for human SHBG [4,31].

In conclusion, the plasma half-life of SHBG is determined primarily by the number of *N*-linked carbohydrate chains rather than their position on the polypeptide backbone. Since the majority of hSHBG is normally comprised of bi-antennary *N*-linked oligosaccharide chains [7] and because the two subunits of a given hSHBG homodimer appear to be glycosylated similarly [32], our data also suggest that hSHBG dimers of the light subunit found in human serum [33] have a relatively longer plasma half-life. Furthermore, the relative amount of this particular glycoform which comprises only a single *N*-linked oligosaccharide on each subunit [8] is increased considerably in human testis homogenates [34] and whether this is due to its synthesis by Sertoli cells, as the androgen-binding protein [34], or is a reflection of preferential uptake of this particular SHBG glycoform from the blood remains to be resolved. If the latter is true, a specific *N*-linked oligosaccharide might represent a signal that directs the extra-vascular compartmentalization of this plasma glycoprotein.

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