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Structure/function analyses of human sex hormone-binding globulin by site-directed mutagenesis

W.P. Bocchinfuso, S. Warmels-Rodenhiser and G.L. Hammond

Departments of Obstetrics and Gynecology, Biochemistry, and Oncology, and MRC Group in Fetal and Neonatal Health and Development, University of Western Ontario, London, Ont. N6A 4L6, Canada

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Human sex hormone-binding globulin (hSHBG) and rat androgen-binding protein (rABP) exhibit distinct affinities for sex-steroids. We therefore constructed and expressed a hSHBG/rABP hybrid cDNA encoding the N-terminal portion of hSHBG (205 residues) and the C-terminal portion of rABP (168 residues). The resulting chimera displayed similar steroid-binding characteristics as hSHBG and was recognised by a monoclonal antibody (S1B5) for hSHBG. We then created substitutions at Ser-133, His-136 and Met-139. The Asp-133 and Gln-136 mutants bound steroids in the same way as normal hSHBG while the steroid-binding affinity of Trp-139 was reduced. All three mutants cross-reacted similarly in a hSHBG radioimmunoassay, but Gln-136 was recognised poorly by the S1B5 antibody. These data imply that residues involved in steroid-binding are located within the N-terminal half of hSHBG and include Met-139, and that the S1B5 epitope is located in this region.

Sex hormone-binding globulin; Androgen-binding protein; Steroid-binding domain; Epitope; Site-directed mutagenesis

1. INTRODUCTION

Human sex hormone-binding globulin (hSHBG) is a plasma glycoprotein that modulates the bioavailability of testosterone and estradiol [1,2]. The rat testis produces a structurally related molecule [3], known as androgen-binding protein (rABP), that binds sex steroids with lower affinity than hSHBG [4]. Affinity-labelling studies of the hSHBG and rABP steroid binding sites have produced inconsistent results [5–10], and it is unclear whether amino acids that interact with steroid ligands are confined to a discrete region surrounding Met-139 [5–7] or are dispersed throughout the molecule [8–10]. To resolve this issue, the primary structures and steroid-binding affinities of SHBG-related molecules in different species [8,11–13] were compared, and this information was used to create a hSHBG/rABP chimera and three hSHBG mutants in which amino acid substitutions were predicted to influence steroid binding. These recombinant proteins were expressed in chinese hamster ovary (CHO) cells, and their steroid-binding characteristics and immunological properties have enabled us to identify amino acids involved in steroid binding and the epitope for a monoclonal antibody against hSHBG [14].

Abbreviations: hSHBG, human sex hormone-binding globulin; rABP, rat androgen-binding protein; CHO, chinese hamster ovary; DCC, dextran-coated charcoal; DHT, 5α-dihydrotestosterone; PAGE, polyacrylamide gel electrophoresis.

Correspondence address: G.L. Hammond, London Regional Cancer Centre, 790 Commissioners Road East, London, Ont. N6A 4L6, Canada. Fax: (1) (519) 685-8616.

2. MATERIALS AND METHODS

2.1. *Construction and expression of a hSHBG/rABP chimera and hSHBG mutants*

A cDNA for the hSHBG precursor [15] was sub-cloned into the *Not*I site of a modified pBluescript vector (Stratagene, La Jolla, CA) that lacks an *Eco*RI site. This construct was digested with *Eco*RI to remove 553 base pairs (bp) from the 3′ end of the hSHBG cDNA, and this was replaced with the corresponding 3′ rABP cDNA fragment [3]. The hybrid cDNA was inserted into the *Not*I site of pRc/CMV (Invitrogen, San Diego, CA) for expression, as described previously [15]. After selection in the presence of G418, stably transfected cells were washed in phosphate-buffered saline to remove fetal calf serum, and were cultured in Dulbecco's modified Eagle medium for 2 days.

For site-directed mutagenesis, the hSHBG cDNA was inserted into a *Hin*dIII/*Xba*I-digested pSelect-1 vector (Promega Corp., Madison, WI), and mutated using oligonucleotide primers to convert the codons for Ser-133(AGC)→Asp(GAC), His-136(CAT)→Gln(CAA), and Met-139(ATG)→Trp(TGG) using the protocol recommended by Promega. The mutated cDNAs were sequenced to confirm that only targeted mutations had occurred, and then inserted into *Hin*dIII/*Xba*I-digested pRc/CMV for expression in CHO cells [15].

2.2. *Measurements of steroid-binding characteristics and immunochemical analyses*

The steroid-binding capacity of recombinant proteins in medium from CHO cell cultures was determined by saturation analysis using [^{3}H]5α-dihydrotestosterone (DHT) as labelled ligand and dextran-coated charcoal (DCC) to separate bound and free steroid [16]. The apparent dissociation rate of protein-bound [^{3}H]DHT was also assessed by exposure to DCC for increasing time at 0°C [16], and affinity constants were measured by Scatchard analysis [16,17]. Relative binding affinities were also determined using [^{3}H]DHT as labelled ligand and increasing amounts of DHT, testosterone, and 17β-estradiol as

competitors [16]. The immunochemical properties of recombinant proteins were examined using an immunoradiometric assay [18] that relies on a monoclonal antibody (S1B5) specific for hSHBG [14], and a radioimmunoassay to assess their ability to compete with [^{125}I]hSHBG for a polyclonal antiserum [18]. Samples of culture medium (15 μl) were also subjected to polyacrylamide gel electrophoresis (PAGE) and Western analysis, as described previously [15].

3. RESULTS AND DISCUSSION

Human SHBG and rABP both comprise 373 amino acids and exhibit 68% sequence identity [3]. Their cDNAs contain a conserved internal *Eco*RI site and this enabled us to conveniently create a hybrid cDNA encoding a hSHBG/rABP chimera (Fig. 1). Since rABP has a lower affinity for androgens than hSHBG [4], and amino acids in both halves of hSHBG have been affinity-labelled [5–10], we predicted that analyses of this chimera would help determine whether the C-terminal region plays a direct role in steroid binding. When expressed in CHO cells, the apparent dissociation rate (Fig. 2A), steroid-binding affinity (Fig. 2B), and specificity (relative binding affinity: DHT = 1.00, testosterone = 0.18, estradiol = 0.05) of the chimera were similar to normal hSHBG [15], and this is remarkable because 35% of the residues in the C-terminal portion of the chimera are specific for rABP (Fig. 1). Thus, although conserved residues in the C-terminal portions of hSHBG and the chimera may be necessary for appropriate folding and dimerization, these data suggest that amino acids within the N-terminal half of hSHBG are responsible for its unique steroid-binding properties.

To further investigate the N-terminal portion of hSHBG, its primary structure was compared with

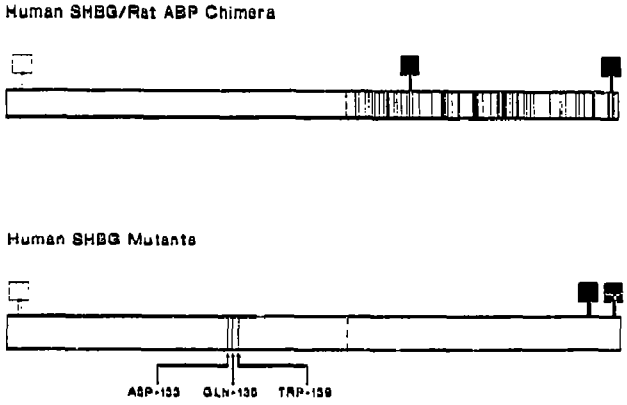

Fig. 1. Diagram of the hSHBG/rABP chimera and hSHBG mutants. Both proteins comprise 373 amino acids. Vertical lines indicate positions of rABP-specific amino acids within the chimera, and the 3 amino acids in hSHBG altered by site-directed mutagenesis. The conserved *Eco*RI site in the hSHBG [8] and rABP [3] cDNAs encompasses the codons for Glu-205 and Phe-206, and its relative position is shown as a broken vertical line. Closed boxes indicate the positions of *N*-linked oligosaccharide chains at residues 244 and 367 in the chimera, and 351 and 367 in the hSHBG mutants, while the *O*-linked oligosaccharide at position 7 is shown as an open box.

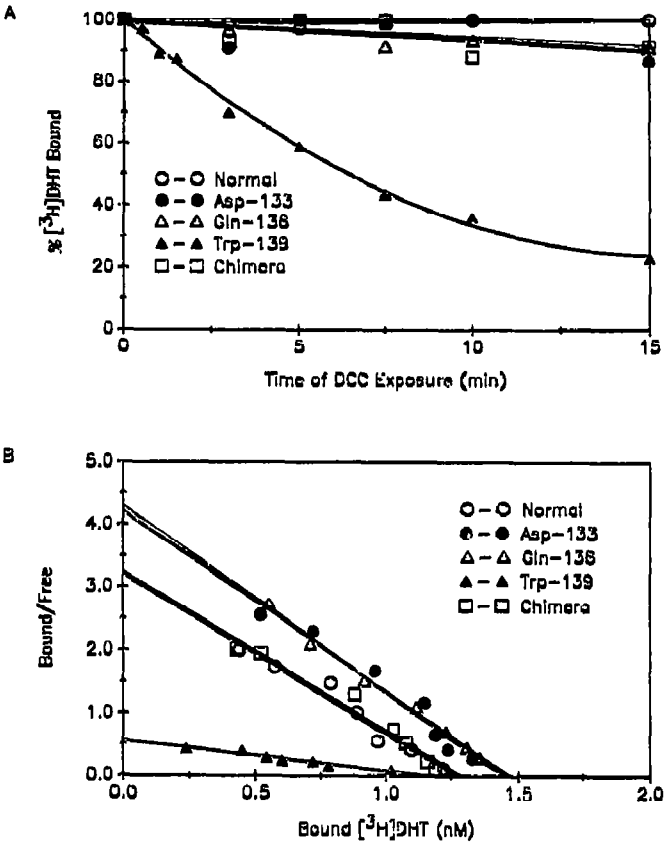

Fig. 2. Measurements of the apparent dissociation rate measurements (A) and Scatchard analysis (B). After 10 min exposure to dextran-coated charcoal (DCC) at 0°C, the dissociation of [^{3}H]DHT from the Trp-139 mutant was 67%, but was <5% for the remaining recombinant proteins (A). Dissociation constants are: 0.40 nM for normal hSHBG; 0.34 nM for Asp-133; 0.35 nM for Gln-136; 0.40 nM for the hSHBG/rABP chimera, and 2.08 nM for Trp-139 (B).

SHBG-related proteins in other mammalian species that resemble rABP in terms of their steroid-binding properties [4,12,19]. This revealed a sequence of poorly conserved polar amino acids adjacent to a conserved hydrophobic region (Fig. 3). Included within this region is a conserved residue (Met-139) that has previously been affinity-labelled in hSHBG [5], and when this was substituted by tryptophan the resultant mutant displayed a marked reduction in steroid-binding affinity, as assessed by both dissociation rate (Fig. 2A) and Scatchard analyses (Fig. 2B). Based on the phylogenetic

	131															140									149
HUMAN	L	T	S	K	R	H	F	I	M	R	I	A	L	G	G	L	L	F	P						
RAT	-	A	D	H	P	Q	L	S	-	-	-	-	-	-	-	-	-	L	-						
MOUSE	-	A	D	H	S	Q	R	S	-	-	-	-	-	-	-	-	-	L	-						
RABBIT	-	H	D	-	P	Q	-	V	-	K	L	-	V	-	-	-	-	-	-						

Fig. 3. Phylogenetic comparison of SHBG-related protein sequences between residues 131–149. Amino acids identical to hSHBG are shown as dashes. Amino acids within the boxed areas were targets for site-directed mutagenesis.

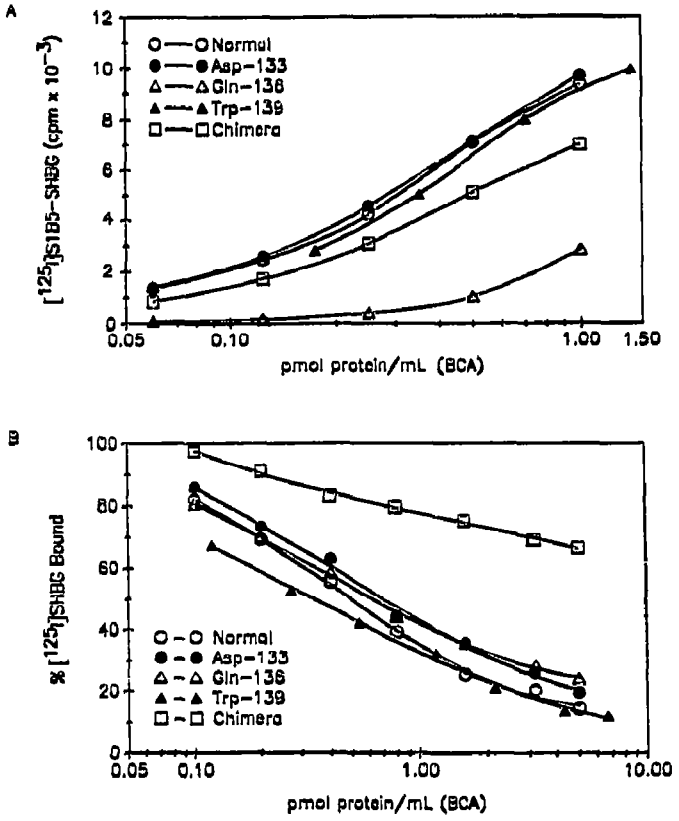

Fig. 4. Analyses of normal hSHBG, the hSHBG/rABP chimera and hSHBG mutants by (A) immunoradiometric assay and (B) radioimmunoassay. A steroid-binding capacity assay (BCA) was used to define the amounts of protein analysed.

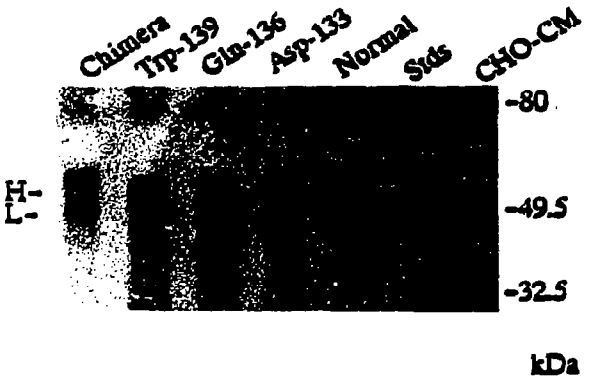

Fig. 5. Western blot of normal hSHBG, the hSHBG/rABP chimera and hSHBG mutants. Proteins were resolved by 7.5% PAGE under denaturing and reducing conditions, electrophoretically transferred to a nylon membrane, and detected immunochemically [15]. Conditioned medium from chinese hamster ovary cells (CHO-CM) represents a negative control, and the positions of size standards (stds) are shown on the right. The positions of the heavy (H) and light (L) subunits typical of hSHBG are shown on the left.

comparison of SHBG-related protein sequences within this region (Fig. 3), we also converted Ser-133 and His-136 in hSHBG, independently, to Asp-133 and Gln-136, because these residues are conserved in rat, mouse and rabbit ABP (Fig. 3). However, the steroid-binding affinities (Fig. 2) and specificities (data not shown) of these mutants were indistinguishable from those of normal hSHBG [15]. Two independent immunochemical approaches were used to demonstrate that the tertiary and quaternary structures of the Trp-139 mutant were not markedly altered. In a two-site immunoradiometric assay based on the use of a single monoclonal antibody that recognises a conformational epitope on both subunits of hSHBG [20], the Trp-139 and Asp-133 mutants showed very similar immuno-reactivity to normal hSHBG, while the Gln-136 mutant reacted poorly with this antibody (Fig. 4A). However, all three mutants displayed similar cross-reactivity with hSHBG in a radioimmunoassay (Fig. 4B) that relies on the use of a polyclonal antiserum against hSHBG [18]. These data demonstrate that the decreased steroid-binding affinity of the Trp-139 mutant is not due to a gross alteration in protein structure. It also appears that Gln-136 disrupts the S1B5 epitope without influencing steroid binding (Fig. 4A), or the overall conformation of the molecule as assessed by the integrity of epitopes recognised by a polyvalent antiserum (Fig. 4B). The sensitivity of this type of immunochemical approach for the detection of structural alterations is illustrated by the poor cross-reactivity of the chimera in the radioimmunoassay (Fig. 4B), and obviously reflects a loss of epitopes within the C-terminal portion of the molecule. However, the chimera cross-reacts significantly with the S1B5 antibody, and this supports the concept that its epitope is confined largely to the N-terminal portion of the molecule.

The chimera and all three mutants were secreted in approximately equal amounts, as assessed by steroid-binding capacity, immuno-reactivity and Western blotting (Fig. 5). The recombinant forms of SHBG migrate with mobilities identical to normal hSHBG during PAGE under native conditions [15], and this is indicative of dimer formation (data not shown). Furthermore, the hSHBG mutants displayed the typical size heterogeneity associated with hSHBG subunits [15] when subjected to PAGE under denaturing conditions (Fig. 5). The chimera also resolved into subunits with slightly lower mobilities than the typical heavy and light isoforms of the hSHBG monomers when analysed in this way (Fig. 5). This probably reflects the addition of more complex carbohydrate structures on both consensus sites for *N*-glycosylation within the chimera (Fig. 1). However, this does not affect steroid binding, and carbohydrates attached to the non-conserved *N*-glycosylation sites do not therefore appear to be involved in this process.

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