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SEX HORMONE-BINDING GLOBULIN: ANATOMY AND PHYSIOLOGY OF A NEW REGULATORY SYSTEM

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Summary—Sex hormone-binding globulin (SHBG) is a plasma glycoprotein that binds a number of circulating steroid hormones (testosterone, dihydrotestosterone and estradiol) with high affinity, thus regulating their free concentration in plasma. In addition to binding steroids, SHBG itself binds to receptor sites on plasma membranes with somewhat unusual kinetics. Both the off and on rates are quite slow. The steroid-binding and membrane-binding functions are intertwined in what is clearly an allosteric relationship. Occupation of SHBG's steroid-binding site by a steroid inhibits its ability to bind to its membrane receptor-binding site. This inhibition is not related to a steroid's biological activity. Metabolites of steroids without biological activity, e.g. 2-methoxyestradiol, actively inhibit SHBG's interaction with its membrane receptor. However, if unliganded SHBG is allowed to bind to its receptor on intact cells, and an appropriate steroid hormone then is introduced, adenylate cyclase is activated and intracellular cAMP increases. This function is specific for steroids with biological activity, 2-methoxyestradiol has no activity in this arena. These observations demonstrate a potentially important role for SHBG as a regulator of cell function. They also demonstrate an additional mode of action of steroid hormones, one that does not require that the steroid interact with a steroid receptor.

INTRODUCTION

The binding of steroid hormones to plasma proteins plays an important role in their transport, distribution and biology. In human plasma, these hormones are bound to the specific steroid hormone-binding proteins, sex hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG); only a small fraction is unbound [1–5]. Until quite recently, it has been generally accepted that the sole function of these proteins is to regulate the concentration of free steroids in plasma. However, the discovery of specific SHBG and CBG receptors in cell membranes from a variety of tissues suggested a broader role for these glycoproteins. During the last few years SHBG has been shown to bind specifically to the plasma membrane of human prostate [6] and decidual endometrium [7–9], as well as to soluble extracts of prostatic membranes [10]. CBG has been shown to bind to human prostate membranes [11], placental syncytiotrophoblast membranes [12] and decidual endometrial membranes [13], as well as to several membranes prepared from a number of tissues from the rhesus monkey [11], and rat [14–17]. These observations suggest that these proteins do something more than regulate the plasma concentration of free steroids. This suggestion is supported by the observation of an increase in adenylate cyclase activity and the accumulation of cAMP following the addition of CBG to MCF-7 cells [18] or SHBG to LNCaP cells [19].

In this communication we will examine what is currently known of the structure and function of the steroid–SHBG (SHBG–receptor) system.

PHYSIOLOGY

SHBG binds to a receptor on prostatic cell membranes

In 1985 [6] we described the binding of [^{125}I]SHBG to human prostatic membranes. Binding was both time and temperature dependent. Further, other human proteins did not compete with [^{125}I]SHBG for binding to the high affinity site. At 37°C, binding at 8 and 24 h was reduced to 80 and 60%, respectively of that at 6 h; therefore, equilibrium binding studies, i.e. Scatchard analyses, were done for 6 h at 37°C.

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Because of the lability of the receptor while contained in the membrane, we did not show the reversibility of the binding in our original communication, but conducted Scatchard analyses on the assumption that reversibility would eventually be shown. This assumption proved to be correct (see below) [10]. In contrast to events at 37°C, binding did not diminish after 24 h of incubation at 4°C. However, specific binding of [^{125}I]SHBG after 24 h at 4°C was only 10% of that observed at 37°C after 6 h. Thus, either equilibrium was not close to being attained, or remarkably fewer sites were accessible to [^{125}I]SHBG at that time and temperature; experiments at 4°C were not pursued. These details assume greater importance when our work is compared to other studies with which there are some discrepancies. Strel'chyonok *et al.* [9] showed that [^{125}I]SHBG bound specifically and with high affinity to human decidual endometrium plasma membranes. However, they studied binding at 4°C at a single time, 16 h [9]. They observed a dissociation constant more than three orders of magnitude less than our current estimates.

The two most obvious ways to account for this discrepancy are a tissue and/or a temperature difference. To analyze one of these possibilities, we again examined the time course of binding of [^{125}I]SHBG to both the membrane-bound and the soluble SHBG receptor at 37°C and compared it to binding at 4°C (Fig. 1). At 4°C, it requires about 48 h to achieve steady state binding to prostatic membranes. Even at 4°C, however, there is an obvious and large loss of binding activity starting at 96 h. Consideration must be given to the possibility that denaturation of the receptor begins much earlier. Otherwise, binding activity should have continued to increase between 48 and 96 h rather than reaching an apparent plateau. Restated, the binding plateau could represent the net result of the binding reaction and receptor denaturation. In analogy to events at 37°C, after 10 days at 4°C binding to the soluble receptor was continuing to increase linearly. However, at that time it still had achieved only 15% of the binding seen at 37°C after 36 h. It is reasonable to presume that, if the receptor were sufficiently stable, binding eventually would have reached the same value as that observed at 37°C. Thus, at least in prostatic tissue, there appears to be internal consistency. The membrane-bound receptor is labile at both 4 and 37°C, very much more so at 37°C. In spite

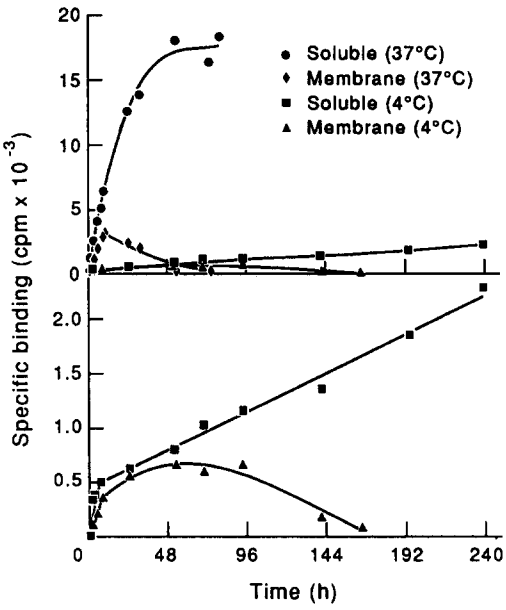

Fig. 1. Time-course of the binding of [^{125}I]SHBG to the membrane-bound and solubilized prostatic SHBG receptor at 4 and 37°C. At time = 0, prostatic membranes or CHAPS-solubilized prostatic receptor preparations were added to flasks that contained [^{125}I]SHBG (about 1.5 nM, 160,000 cpm/0.25 ml), $\pm 2.2\ \mu$M radioinert SHBG. At the indicated times, 250 μl aliquots, in triplicate, were removed and binding was determined. Specific binding is the difference between total binding ([^{125}I]SHBG only) and non-specific binding (binding in the presence of excess cold SHBG). The methods for preparation of the membranes, solubilization of the receptor, and the measurement of binding were as described previously [6, 10]. The lower half of the figure shows the data at 4°C (from the upper panel) on an expanded scale.

of the greater degree of stability of the membrane bound receptor at 4°C, the binding of [^{125}I]SHBG that ultimately is achieved is only a fraction of that at 37°C because of the huge difference in the rate of binding of [^{125}I]SHBG at these two temperatures, Fig. 1.

Although the membrane bound receptor was unstable, the soluble one proved to be remarkably hardy. After 100 h at 37°C or 240 h at 4°C, there was no loss of binding activity ([10], and Fig. 1). Finally, the stability of the soluble receptor permitted the demonstration that the binding reaction was reversible (Fig. 2), as we had assumed but not proved. Thus, all the conditions for obtaining equilibrium constants were met. As observed with prostatic membranes, a high affinity and a low affinity site were present in the soluble receptor [10]. In addition, the high affinity dissociation constant determined at equilibrium was examined independently by determining the quotient (k_2/k_1) of the reverse (k_2) and forward (k_1) rate constants. The agreement between these two independent

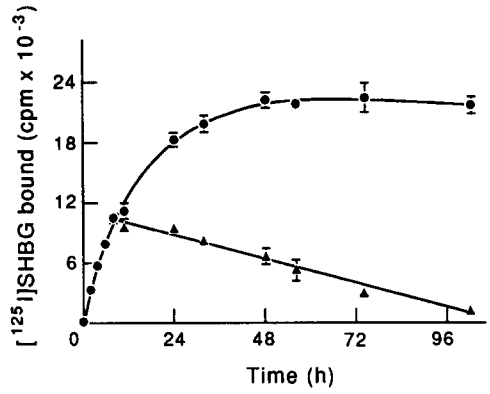

Fig. 2. Time-course of association and dissociation of [^{125}I]SHBG binding to the solubilized prostatic SHBG receptor at 37°C. At time = 0, CHAPS-solubilized prostatic receptor preparations were added to each of 3 flasks, all of which contained [^{125}I]SHBG (1.55 nM, about 166,000 cpm/0.25 ml), and one of which contained 2.2 μM radioinert SHBG. At the indicated times, 250 μl aliquots, in triplicate, were removed and binding was determined (●). Specific binding is the difference between total binding ([^{125}I]SHBG only) and non-specific binding (binding in the presence of excess cold SHBG). After 8 h, one of the flasks containing [^{125}I]SHBG alone was made 2.2 μM in radioinert SHBG, and the dissociation of [^{125}I]SHBG from its receptor was followed (▲). Each data point is the mean ± SD of triplicates (absence of an error bar indicates an error which is smaller than the point). A semi-log plot of the dissociation of [^{125}I]SHBG vs time was linear, and the dissociation rate constant (k_2) was determined from the slope of that line (not shown). In several similar experiments, 6–8% of the added [^{125}I]SHBG was specifically bound to the soluble receptor after 6–8 h and 8–12% after 24–48 h at 37°C. Non-specific binding was constant after 2 h and represented 35% of total binding at 6–8 h, and about 25% of total binding at 24 h and thereafter. (Reproduced with permission from Hryb *et al.* [10].)

approaches toward obtaining the dissociation constant was excellent. The average K_d, using both methods, was 0.65 nM [10]. This K_d is about 10-fold less than that seen when using prostatic membranes rather than the soluble receptor [6]. The phenomenon of increased affinity subsequent to receptor solubilization has been reported for the interaction of prolactin and its receptor [20].

The effect of steroids on the interaction of SHBG with its receptor

None of the work in the preceding section examined the effect of steroids on the SHBG–receptor interaction. Strel'chyonok *et al.* [9] had reported that only estradiol-liganded SHBG was able to bind to decidual endometrium membranes at 4°C; testosterone-bound SHBG or unliganded SHBG did not bind to these membranes. We undertook to examine the effect of a variety of steroids on the solubilized prostatic receptor at 37°C [21]. Much to our surprise, no SHBG–steroid complexes bound to the SHBG receptor; only unliganded SHBG did. Figure 3 illustrates the effect of six different steroids on the binding of unliganded SHBG to its receptor. The comparative inhibitory potency of this group of steroids is in exactly the same sequence as the tightness of their association with SHBG [22–24]. The non-competitive nature of the inhibition is shown by the double reciprocal plot in Fig. 4. That the inhibitory effect of steroids was mediated by their binding to SHBG, rather than to the SHBG receptor, was ascertained by showing that dihydrotestosterone (DHT) did not bind to the receptor [21]. Thus, SHBG is an allosteric protein whose membrane-binding site is obscured when its steroid-binding site is occupied.

The foregoing formulation does not deal with the ability of receptor-bound SHBG to bind steroids. The fact that occupation of SHBG's steroid-binding site masks its membrane receptor-binding site does not imply the converse. That is, it is not necessary that occupation of SHBG's membrane receptor-binding site obscure its steroid binding site. Indeed, this is not the case. Unliganded SHBG that was bound to the soluble receptor was fully capable of binding DHT (Fig. 5). Although occupancy of the steroid-binding site resulted in dissociation of SHBG–DHT from the receptor–SHBG–DHT complex, the rate of dis-

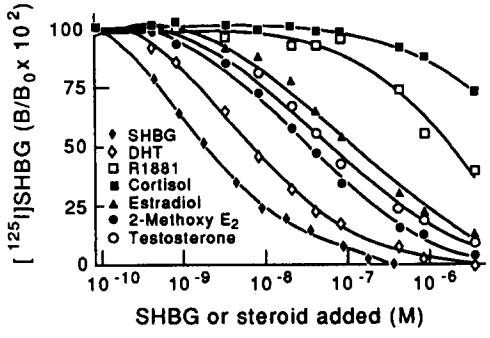

Fig. 3. The effect of radioinert SHBG and a variety of steroids on the binding of [^{125}I]SHBG to its solubilized receptor. [^{125}I]SHBG (0.5–1.0 nM, about 180,000 cpm) ± radioinert SHBG, or the indicated steroid, was added to the solubilized SHBG receptor and allowed to incubate at 37°C for 40 h, after which bound [^{125}I]SHBG was precipitated with PEG 8000 and counted. B_o, Specific binding of [^{125}I]SHBG in the absence of competitor (6–12% of the added cpm); B, Specific binding of [^{125}I]SHBG in the presence of competitor. Non-specific binding was 25–35% of total binding. Data are shown as the mean of duplicate determinations for each curve. E_2, estradiol; R1881, methyltrienolone. (Reproduced with permission from D. J. Hryb *et al.* [21].)

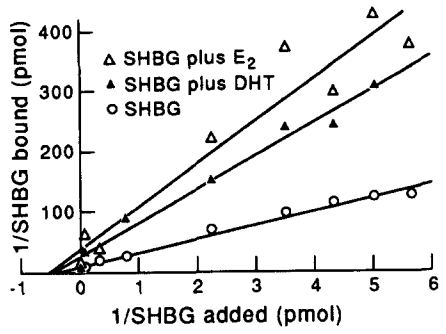

Fig. 4. Double reciprocal plot—non-competitive inhibition of the binding of [^{125}I]SHBG to its receptor by estradiol and DHT. [^{125}I]SHBG (0.7 nM, about 190,000 cpm) was incubated with the solubilized prostatic receptor in the presence of increasing amounts of radioinert SHBG (0–0.4 μM) (SHBG) in the absence or presence of a constant amount of estradiol (5×10^{-8} M) (SHBG plus E_2) or DHT (4×10^{-9} M) (SHBG plus DHT). Incubations were carried out at 37°C for 40 h, after which bound [^{125}I]SHBG was precipitated [10] and the pellet counted.

sociation was equal to the intrinsic k_2 of the complex, 3×10^{-4} min^{-1} ($t_{1/2} = 39$ h) [21].

Once again these data are in partial but important disagreement with that of Strel'chyonok *et al.* [9, 25] who found that SHBG bound to its receptor in the presence of estradiol but that unliganded SHBG, or SHBG bound to an androgen, could not bind. Although we agree that androgens inhibit receptor binding, we confirmed neither the inability of unliganded SHBG to bind, nor the capacity of estradiol to enhance binding. Once again, their experiments were done at 4°C using a different tissue from us. However, it would appear that if the model of steroid-enhanced binding has generality, then one might expect a classic androgen target tissue, i.e. prostate, to have shown enhanced binding of SHBG in the presence of DHT. This was not the case. We found that *all* steroids that bound to SHBG inhibited its receptor binding. Clearly this statement is speculative; the possibility remains that, by a variety of possible mechanisms, tissue specific differences may exist. In any event, our current concept of the binding system is set out in Fig. 6.

Consequences of the interaction of SHBG with cells

To explore the consequences of SHBG's interaction with its receptor we chose to use the human prostatic carcinoma cell line, LNCaP, which is known to have androgen but not estrogen receptors [26]. The major result of that exploration [19] is shown in Fig. 7. In brief, we exposed LNCaP cells to a sufficient concentration of unliganded SHBG to occupy their receptor sites in 1 h. Unbound SHBG then was washed out of the system and the appropriate vehicle or steroid was added to the cell suspension. Fifteen minutes later, intracellular cAMP was measured. Unliganded SHBG alone had a minimal stimulatory effect. DHT alone was without effect. However, when either DHT or estradiol was added to cells whose receptors were occupied by SHBG, there was a dose dependent increase in intracellular cAMP. 2-Methoxyestradiol, which is a biologically inactive metabolite of estradiol, but which is more potent than estradiol in inhibiting the receptor binding of SHBG (Fig. 2), did not cause an increase in intracellular cAMP. Thus, although the inhibition of the receptor binding of SHBG by steroids is somewhat non-specific, the induction of adenylate cyclase activity appears to be limited to steroids with biological activity. In a more speculative vein, one might envision a use for even "inactive" steroid metabolites. Such metabolites could function to inhibit the binding of "active" steroids to the SHBG–receptor complex and thus assist in the regulation of effects distal to the generation of cAMP.

ANATOMY

In 1986 Walsh *et al.* [27] published the primary structure of human SHBG. By the following year the published sequence was

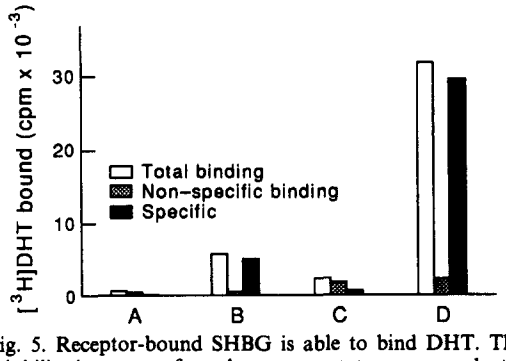

Fig. 5. Receptor-bound SHBG is able to bind DHT. The solubilized receptor from human prostates was covalently coupled to Affi-gel (Affi-gel-receptor). Affi-gel alone (B) and Affi-gel-receptor (D) were incubated with 85 nM unliganded SHBG after which the gels were washed to remove unbound SHBG. Affi-gel (A) and Affi-gel-receptor (C) not exposed to SHBG served as controls. The ability of DHT to bind to SHBG that was bound to receptor was evaluated by incubating the gels with [^{3}H]DHT (10^5 cpm, 8 nM) ± 200-fold radioinert DHT. Free DHT was removed by washing and centrifuging the gels, after which bound [^{3}H]DHT was ascertained. (Adapted from Table II, Hryb *et al.* [21].)

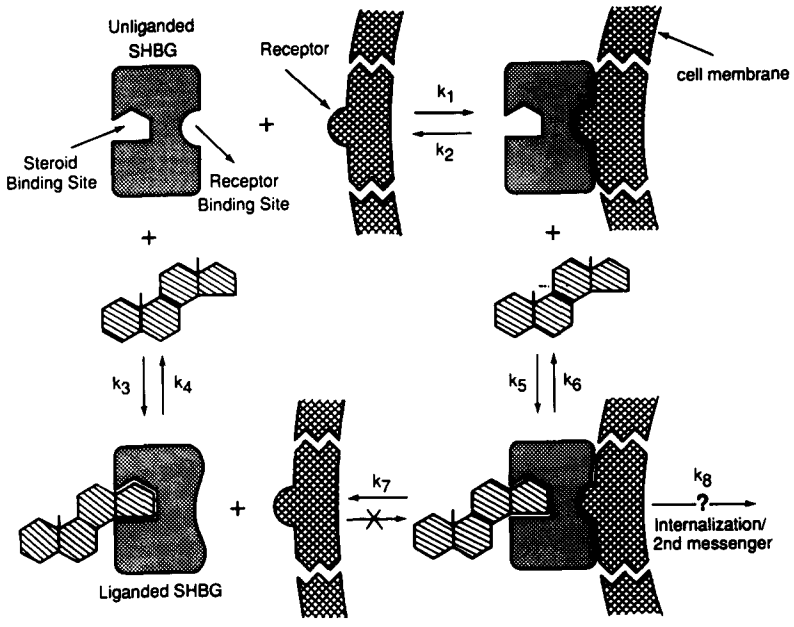

Fig. 6. Model of the receptor–SHBG–steroid system. SHBG has two binding sites, one binds steroids, and the other binds to a membrane receptor. It is an allosteric protein which undergoes a conformational change, resulting in masking of the receptor binding site when a steroid occupies the steroid-binding site. After unliganded SHBG binds to the membrane receptor, it maintains the ability to bind steroids. This does not cause an immediate dissociation of the steroid–SHBG complex from the membrane receptor. Rather, if the steroid which binds to the pre-formed SHBG–receptor complex is appropriate then intracellular cAMP accumulation results (see Fig. 7). (Reproduced with permission from D. J. Hryb *et al.* [21].)

reaffirmed from the deduced nucleotide sequence of its cloned cDNA [28–30]. The direct analysis of SHBG's sequence also ascertained the location of the two N-linked and single oligosaccharide chains whose presence and composition were described three years previously [31]. It is the heterogeneity in the glycosylation of SHBG's two identical subunits that give rise to the distinct subunits observed when SHBG is subjected to gel electrophoresis under denaturing conditions [32]. All this important structural work set the stage for an exploration of the various active regions of the molecule.

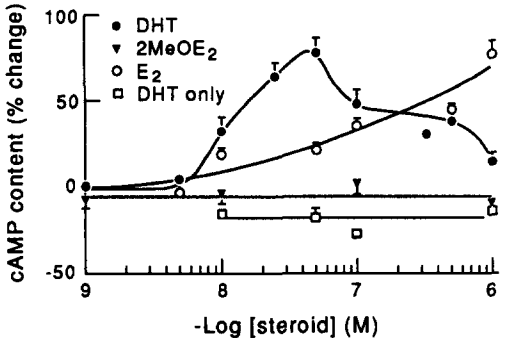

Fig. 7. Effect on cAMP of adding steroids to LNCaP cells to which unliganded SHBG was already bound. LNCaP cells were placed in serum free medium and then incubated for 1 h at 37°C with 50 nM unliganded SHBG. The cells were washed once with serum free medium (to remove free SHBG), and then incubated for 15 min in serum free medium, containing the indicated concentration of steroid and isobutylmethylxanthine (0.1 mM). Results are presented as the percent change from cells incubated with only unliganded 50 nM SHBG. The percent increase in cAMP after adding 50 nM unliganded SHBG, as compared to the addition of buffer, was $7.8 \pm 3.8\%$. The results are the means from 3 experiments conducted in duplicate. DHT only, no preincubation with SHBG. Basal cAMP = 211 ± 19 pmol/mg protein ($n = 15$). $2MeOE_2$, 2-methoxyestradiol; E_2, estradiol. (Reproduced with permission from A. M. Nakhla *et al.* [19].)

Purified human SHBG has been photoaffinity labeled with Δ^6-[^{3}H]testosterone by two different groups of investigators [28, 33]. After labeling, both groups submitted the labeled SHBG to tryptic digestion and then separated the tryptic peptides by reverse phase HPLC. Hammond *et al.* [28] isolated a labeled tryptic peptide which indicated that "a portion of the steroid-binding domain of SHBG is located between residue 296 and the 35 predominantly hydrophobic residues at the C-terminus of the protein". Surprisingly, the experiments of Grenot *et al.* [33] indicated that the photoaffinity label, Δ^6-[^{3}H]testosterone, attacked the methionyl residue at position 139. These discrepant results await clarification.

We also used the approach of affinity labeling to start to identify the steroid-binding site of

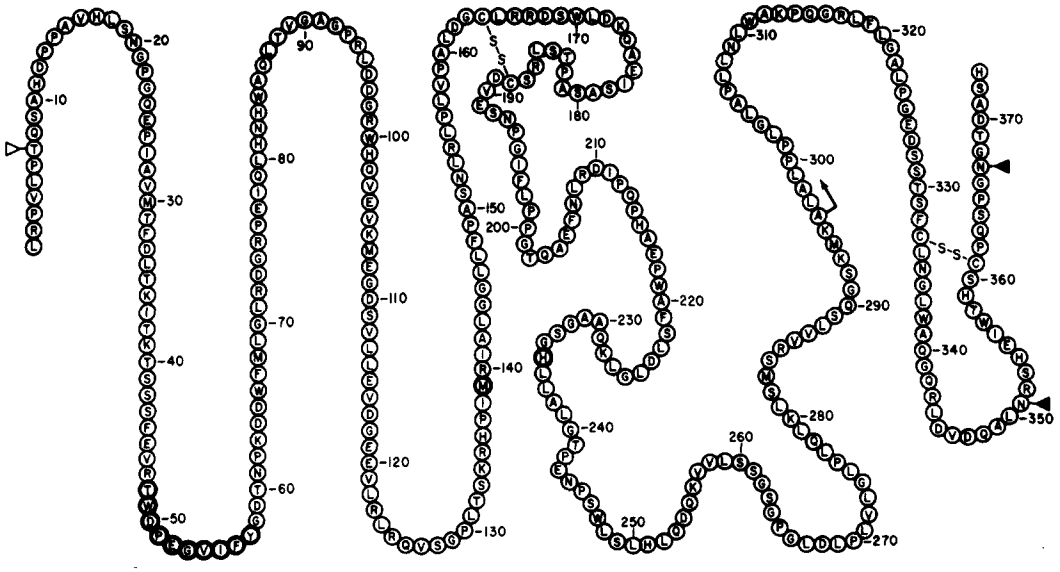

Fig. 8. The primary structure of human SHBG. Histidine 235 was identified in the steroid binding site by affinity labeling with 17β-bromoacetoxydihydrotestosterone [35]. Δ^6-Testosterone was used to identify either the peptide distal to alanine 296 [28] or methionine 139 [33] as being in the steroid-binding site. The decapeptide extending from residue 48 to residue 57 constitutes a portion of SHBG's membrane receptor binding domain [36]. The sites of attachment of SHBG's two N-linked oligosaccharides and its single O-linked oligosaccharide are identified by closed and open triangles, respectively.

SHBG. However, we used a nucleophilic derivative of DHT, 17β-bromoacetoxyDHT, which had been shown previously to affinity label 3α,20β-hydroxysteroid dehydrogenase [34]. After demonstrating that [^{14}C]17β-bromoacetoxyDHT fulfilled the criteria for an affinity labeling compound, SHBG was affinity labeled with it and then submitted to acid hydrolysis. The released amino acids were evaluated on HPLC, and virtually all of the ^{14}C was identified as 3-[^{14}C](carboxymethyl)histidine. Further, [^{14}C]17β-bromoacetoxyDHT-labeled SHBG was digested with trypsin, followed by isolation of the released tryptic peptides by reverse phase HPLC. The ^{14}C was localized to a single tryptic peptide. It contained two histidyl residues, corresponding to residues 235 and 251 in the amino acid sequence of SHBG. Although most of the 3-[^{14}C](carboxymethyl)histidine, or its phenylthiohydantoin derivative, was trapped on the filter of the amino acid sequencer, sufficient radioactivity emerged to identify histidyl residue 235 as the labeled amino acid [35]. A summary of the areas identified as candiates for the steroid binding domain of SHBG is shown in the cartoon in Fig. 8. Correlation of these areas with SHBG's tertiary structure will be needed to arrive at a final understanding of its steroid-binding domain.

As indicated previously, SHBG must also have a membrane receptor-binding domain that is different from its steroid-binding domain. We, therefore, undertook studies to identify, isolate, sequence and synthesize the region of SHBG that interacts with its membrane receptor [36]. To accomplish this, highly purified human SHBG was digested with trypsin. The SHBG-derived tryptic peptides were separated by HPLC and evaluated for their ability to compete with [^{125}I]SHBG for binding to the SHBG receptor. Only a single peptide, corresponding to residues 48–57 of the known sequence of human SHBG, inhibited receptor binding. We synthesized this decapeptide and showed that it inhibited SHBG binding in a manner indistinguishable from the isolated tryptic peptide. The K_d of SHBG(48–57) was very much greater than that of the native SHBG, but this observation was not surprising. Even greater increases in dissociation constants have been seen when examining the receptor-binding region of the α-subunit of human chorionic gonadotropin (hCG) [37]. It is likely that the native receptor-binding region of SHBG involves discontinuous as well as continuous sites. That is, there are undoubtedly important features of SHBG's folded structure that are critical in ensuring maximal binding strength. The average hydrophilicity is greater in the amino terminal portion of SHBG, the location of the receptor-binding domain (Fig. 8), than in the carboxyl terminus. It is likely that segments

of the receptor-binding domain occupy a region of SHBG that is at or near the exterior surface of the molecule. The markedly hydrophobic carboxyl terminus is the site of at least a portion of the steroid-binding domain, and is undoubtedly situated in SHBG's interior. These sites must communicate with one another because, as we have shown, SHBG is an allosteric protein whose receptor binding site is masked by the binding of a steroid at its steroid-binding site.

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