Steroid-Binding and Dimerization Domains of Human Sex Hormone-Binding Globulin Partially Overlap: Steroids and Ca^{2+} Stabilize Dimer Formation†

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ABSTRACT: Human sex hormone-binding globulin (SHBG) is a homodimeric plasma glycoprotein with a single steroid-binding site for biologically active sex steroids, and a methionine at position 139 (M139) interacts with the photoaffinity ligand, Δ^6-testosterone. We have introduced amino acid substitutions into this and other locations in the SHBG molecule and have examined their impact on steroid binding and dimerization. As a result, substitutions at residues 134–139 generate alterations in steroid-binding specificity. In particular, substitutions at residues 134–138 were characterized by altered binding affinities for estradiol relative to 5α-dihydrotestosterone (DHT), and one of them (R135L) also showed a 2-fold increase in affinity for C19 steroids with a 3β-hydroxy group. Unlike all of the other mutants we have examined, the M139W mutant has a 5-fold lower affinity for DHT, and its affinities for testosterone, 5α-androstane-3β,17β-diol, and estradiol also appear to be reduced to a similar extent. By contrast, M139W appears to bind androst-5-ene-3β,17β-diol with only 2-fold less affinity than wild-type SHBG, while its affinity for 19-nortestosterone remains unaffected. Substitutions at other positions, including those immediately C-terminal to M139, had no effect on steroid-binding affinity and/or specificity. These data provide evidence that residues 134–139 influence the recognition of specific A/B ring conformations of steroid ligands and may constitute part of the steroid-binding domain. We have also found that substitutions at residues 138–148 impair dimerization and that this defect may be abrogated by occupancy of the steroid-binding site. The removal of divalent cations also destabilizes dimer formation of mutants with substitutions at residues 140–148, and Ca^{2+} or Zn^{2+}, but not Mg^{2+}, can restore their ability to form dimers. Steroid ligands and divalent cations also appear to act independently to stabilize dimer formation. These data lead us to conclude that the steroid-binding and dimerization domains of human SHBG partially overlap and that steroid ligands and divalent cations influence the dimerization interface to enhance subunit association.

Plasma sex hormone-binding globulin (SHBG[1]) and testicular androgen-binding protein (ABP) are differentially glycosylated products of a gene located on the short arm of human chromosome 17 (Hammond, 1993). They function as homodimers with a single binding site for androgens and 17β-estradiol (Hammond et al., 1986; Danzo et al., 1989), and the requirements for ligand binding to SHBG include a planar steroid molecule with an electronegative moiety at C3 and a β-hydroxyl group at C17 (Cunningham et al., 1981). Details about the orientation of ligands in the binding site and its topography are limited, but studies involving affinity labeling (Grenot et al., 1992) and site-directed mutagenesis (Bocchinfuso et al., 1992; Sui et al., 1992) have indicated that a methionine at position 139 (M139) is probably important for interaction with the steroid B ring. In addition, the steroid-binding characteristics of a human SHBG/rat ABP chimera suggest that residues important for high-affinity steroid binding are situated within the N-terminal 205 amino acids of human SHBG (Bocchinfuso et al., 1992).

A phylogenetic comparison of plasma sex steroid-binding proteins (Hammond, 1993) indicates that their primary structures are poorly conserved immediately N-terminal to M139, and this could account for species differences in their steroid-binding affinity or specificity (Westphal, 1986). Like its counterparts in other species, human SHBG binds 5α-dihydrotestosterone (DHT) with the highest affinity, but these proteins also bind several other steroids with functional groups that may be spatially closer to specific amino acids within their binding sites. Thus, it is conceivable that different steroid ligands, with unique structural properties, may be utilized as molecular probes to evaluate the subtle changes in steroid-binding activity that occur as a result of specific amino acid substitutions.

A region of alternating leucines between residues 267 and 281 in human SHBG has been suggested to contribute to both steroid-binding and dimer stability (Walsh et al., 1986; Petra, 1991). However, there is no experimental evidence to support this, and the structural requirements for SHBG dimerization are unknown. Dimerization of SHBG is also thought to be necessary for the formation of a single steroid-binding site, and ligand binding appears to stabilize the homodimer (Casali et al., 1990).

The steroid-binding activity of SHBG is preserved more effectively during storage in the presence of both androgen and calcium (Rosner et al., 1974). This suggests that interaction with a divalent cation, as well as occupancy of the steroid-binding site, may help maintain its structural integrity. To identify residues involved in the steroid binding and/or dimerization of human SHBG, we have created mutants with

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[1] Abbreviations: SHBG, sex hormone-binding globulin; ABP, androgen-binding protein; DHT, 5α-dihydrotestosterone; DCC, dextran-coated charcoal; CHO, Chinese hamster ovary; T, testosterone; E_2, 17β-estradiol; 5αA, 5α-androstane-3β,17β-diol; Δ5A, androst-5-ene-3β,17β-diol; PAGE, polyacrylamide gel electrophoresis.

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individual amino acid substitutions in a region encompassing M139 and other locations in the protein. Differences in their physicochemical properties have led us to conclude that the steroid-binding and dimerization domains of human SHBG partially overlap.

EXPERIMENTAL PROCEDURES

Construction and Expression of Mutant SHBG cDNAs. A cDNA for the human SHBG precursor (Bocchinfuso et al., 1991) was inserted into a *Hin*dIII/*Xba*I-digested pSelect-1 vector (Promega) and mutated using oligonucleotide primers, as recommended by Promega. The choice of amino acid substitutions was based primarily on a phylogenetic comparison of published SHBG/ABP sequences (Hammond, 1993), in order to limit structural perturbations of the molecule and because defined differences in SHBG/ABP steroid-binding affinity and specificity exist between species (Westphal, 1986). At positions where residues are invariably conserved between species, amino acid substitutions were initially made to produce charge changes, but when these resulted in a loss of secretion, more conservative substitutions were introduced. An additional mutant was created in which leucines at positions 275, 277, 279, and 281 were all substituted with isoleucine. The mutated cDNAs were sequenced (Sanger et al., 1977), to confirm that only the targeted mutations had occurred, and inserted into a *Hin*dIII/*Xba*I-digested pRc/CMV vector (Invitrogen) for expression in Chinese hamster ovary (CHO pro$^-$ wild-type) cells (Bocchinfuso et al., 1991). After selection in the presence of the neomycin analog, geneticin (Gibco BRL), stably transfected cells were washed in phosphate-buffered saline to remove fetal calf serum and then cultured in Dulbecco's Modified Eagle medium containing 100 nM 5α-dihydrotestosterone (DHT) for two days.

Steroid-Binding Assays. Steroid-binding measurements (Hammond & Lähteenmäki, 1983) were performed on recombinant SHBGs secreted into the culture medium. Samples containing ~1 nM SHBG were incubated (2 h at room temperature) with 10 nM [^{3}H]DHT (43.7 Ci/mmol, Dupont Canada) in the presence or absence of 200-fold molar excess of unlabeled DHT to monitor nonspecific binding. Bound and free steroids were separated with a 600-μL slurry of dextran-coated charcoal (DCC) for 10 min at 0 °C. The steroid-binding affinities of the mutant SHBGs were compared initially to wild-type SHBG in a competition assay using predetermined amounts of DHT, testosterone (T), 17β-estradiol (E_2), 5α-androstane-3β,17β-diol (5αA), androst-5-ene-3β,17β-diol (Δ5A), and 19-nortestosterone (19norT), which displaced 50% of the tracer from wild-type SHBG (Hammond & Lähteenmäki, 1983). Those mutants exhibiting affinities for these ligands that differed by more than 3 standard deviations (SD) of the mean values obtained for wild-type SHBG were analyzed further using increasing amounts of the appropriate competitors to generate complete competition curves (Hammond & Lähteenmäki, 1983). The same methods were used to examine the steroid-binding characteristics of the M139W mutant, which binds DHT with 5-fold lower affinity than wild-type SHBG (Bocchinfuso et al., 1992). Approximately 1 nM M139W was incubated with 20 nM [^{3}H]DHT to ensure saturation of the binding site, and bound and free steroids were separated by exposure to DCC for only 5 min at 0 °C. Wild-type SHBG was also examined under the same conditions in these experiments.

Physicochemical Analysis of SHBG Mutants. The effect of amino acid substitutions on the ability of SHBG mutants to dimerize was assessed under different experimental conditions by polyacrylamide gel electrophoresis (PAGE) and Western blotting (Towbin et al., 1979). All samples (10 μL) containing ~1.5 nM SHBG were subjected to PAGE (4% acrylamide stacking gel and 10% acrylamide resolving gel) under nondenaturing conditions, and proteins were transferred electrophoretically from the gel onto an Immobilon-P membrane (Millipore). Western blots were blocked and then incubated with a rabbit antiserum against human SHBG (diluted 1/333). Immunoreactive proteins were detected using an alkaline phosphatase-labeled second antibody (diluted 1/3333), as described previously (Bocchinfuso et al., 1991).

Densitometric Analysis. An UltrascanXL enhanced laser densitometer (LKB) was used to scan a positive photographic image of Western blots. After subtraction of background staining, the amount of immunoreactive monomeric SHBG was expressed as a percentage of total immunoreactive SHBG in each lane.

RESULTS

Steroid-Binding Properties of Human SHBG Mutants. Two of the SHBG mutants (A142E and G144E) were

Table 1: Affinities of Steroid Ligands for SHBG Mutants Relative to Wild-Type SHBG[a]

SHBG mutants[b]	testosterone	5αA-3β, 17β-diol	Δ5A-3β, 17β-diol	19-nortestosterone	17β-estradiol
S133D	=	=	=	=	=
K134H	=	=	=	=	decrease (2×)
R135L	=	increase (1.6×)[c]	increase (1.8×)	=	increase (2×)
H136Q	=	=	=	=	decrease (1.3×)
P137A	=	=	=	=	increase (1.3×)
I138S	=	=	=	=	increase (2.5×)
R140L	=	=	=	=	=
G144A	=	=	=	=	=
G145E	=	=	=	=	=
L146I	=	=	=	=	=
L147K	=	=	=	=	=
F148L	=	=	=	=	=
E176Q	=	=	=	=	=
N193Q	=	=	=	=	=
L198F	=	=	=	=	=
L275–281I	=	=	=	=	=

[a] The SHBG mutants were screened for their relative steroid-binding affinities using predetermined amounts of various competitors that displaced 50% of [^{3}H]DHT from wild-type SHBG. Three separate screens were performed in triplicate for each competitor, and binding affinities were assessed relative to wild-type SHBG. =: Equivalent affinity to wild-type human SHBG (within mean ±3 SD). [b] Mutants A142E, G144E, and L146K were not secreted in sufficient amounts for analysis. [c] Values in parentheses are derived from Figures 1 and 2.

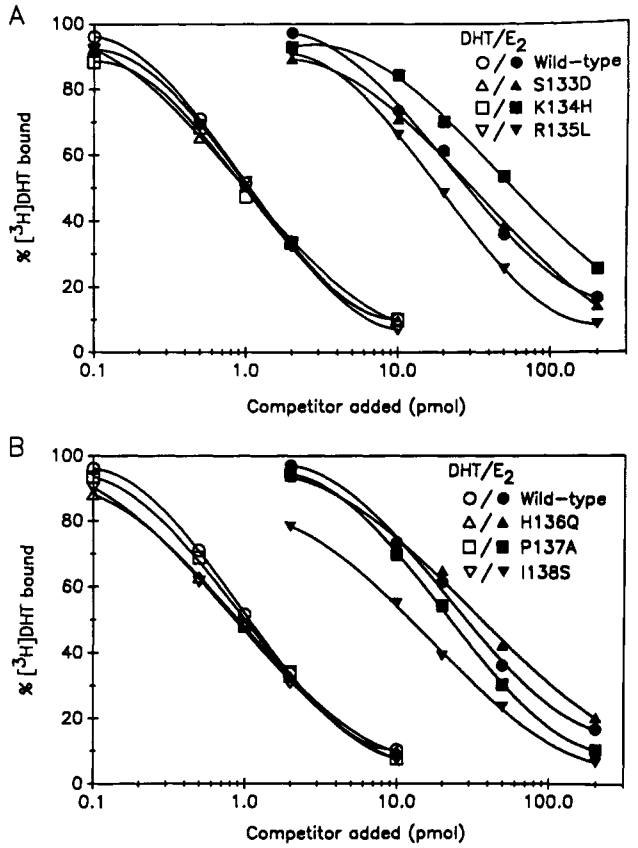

FIGURE 1: Affinities of wild-type and mutant SHBGs for estradiol (E_2) relative to 5α-dihydrotestosterone (DHT). Competition curves were generated by incubating various SHBGs with [^{3}H]DHT and increasing amounts of unlabeled E_2 (closed symbols) or DHT (open symbols). (A) Wild-type SHBG versus SHBG mutants 133–135; (B) wild-type SHBG versus SHBG mutants 136–138.

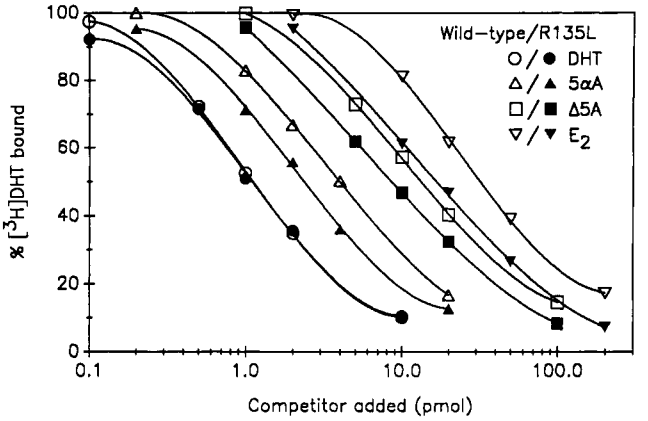

FIGURE 2: Affinities of wild-type SHBG and the R135L SHBG mutant for various steroid ligands relative to 5α-dihydrotestosterone (DHT). Competition curves for wild-type SHBG (open symbols) and the R135L SHBG mutant (closed symbols) were generated by incubation with [^{3}H]DHT and increasing amounts of unlabeled DHT, 5α-androstane-3β,17β-diol (5αA), androst-5-ene-3β,17β-diol (Δ5A), or estradiol (E_2).

Table 2: Affinities of Wild-Type and M139W SHBGs for Steroid Ligands Relative to DHT[a]

competitor steroid	wild-type SHBG (%)	M139W[b] SHBG (%)
5α-dihydrotestosterone	100	100
testosterone	12.0	13.0
5α-A-3β,17β-diol	40.0	33.0
Δ5A-3β,17β-diol	6.7	16.7 (increase 2.5×)
17β-estradiol	3.0	4.0
19-nortestosterone	0.4	2.0 (increase 5×)

[a] The relative binding affinities of wild-type and M139W SHBGs were determined from full competition curves as the amount of competitor steroid required to displace 50% of the labeled ligand ([^{3}H]DHT) compared to DHT. [b] M139W binds [^{3}H]DHT with 5× less affinity than does wild-type SHBG (6).

undetectable in culture medium by Western blotting (data not shown), and one (L146K) was present in trace amounts that precluded further studies. The remainder could be readily detected in the culture medium by a steroid-binding capacity assay in amounts that varied from 0.6 (R140L) to 5.0 nM (F148L). Unlike M139W (Bocchinfuso et al., 1992), all of the other mutants we examined bind [^{3}H]DHT with affinities that are indistinguishable from that of wild-type SHBG on the basis of an analysis of the dissociation rate of labeled ligand during exposure to DCC for increasing times at 0 °C (data not shown). In addition, these mutants all bound T and 19norT with the same affinity as wild-type SHBG (Table 1), while those with amino acid substitutions at residues 134–138 were characterized by abnormal affinities for E_2. Two of them had a reduced affinity for E_2, and this was most pronounced in the case of the K134H mutant (Table 1, Figure 1). Conversely, three of these mutants displayed an increased affinity for E_2, and this was best demonstrated by the I138S and R135L mutants (Table 1, Figure 1). Furthermore, the R135L mutant also had an approximately 2-fold increase in affinity for C19 steroids with a 3β-hydroxy group (5αA and Δ5A), when compared to wild-type SHBG (Table 1, Figure 2). It should be noted that when unlabeled DHT was used as the competitor, the competition curves for these mutants were superimposable with those obtained for wild-type SHBG (Figures 1 and 2), and this also indicates that their affinities for DHT are essentially identical. Substitutions in the region immediately C-terminal to M139 (residues 140–148), and at other locations (residues 175, 193, and 198), had no effect on the steroid-binding properties of SHBG (Table 1).

The steroid-binding characteristics of the M139W mutant were analyzed separately due to its low affinity for DHT. When compared to the affinity of wild-type SHBG for various ligands relative to DHT, the M139W mutant displayed 2.5- and 5-fold greater affinities for Δ5A and 19norT, respectively, while its affinities for T, 5αA, and E_2 were very similar to those associated with the wild-type protein (Table 2).

Influence of Steroid Ligands on Dimerization. The SHBG mutants listed in Table 1 were also assessed for their ability to form stable dimers under different experimental conditions (Figures 3–7). This list included a mutant in which leucines at positions 275, 277, 279, and 281 were substituted with isoleucine (L275–281I), and which also bound steroid with wild-type characteristics (Table 1).

The wild-type and mutant SHBGs were tested initially for dimer formation in the presence or absence of androgen (Figure 3). When steroid was removed from the samples, mutants containing amino acid substitutions at residues 138–148 were characterized by the presence of an immunoreactive monomeric form of SHBG that migrates with an $R_f = 0.50$ (relative to the bromophenol blue dye front) during electrophoresis under nondenaturing conditions. In other samples, the majority of immunoreactive SHBG was associated with an electrophoretic species (R_f = 0.20) that is characteristic of the normal homodimeric form of SHBG. In some samples, particularly those containing SHBGs with substitutions at residues 138–148, a third electrophoretic species was identified (R_f = 0.26). This diffuse electrophoretic form is probably similar in molecular size to the natural dimer and may represent dimeric complexes with altered conformations.

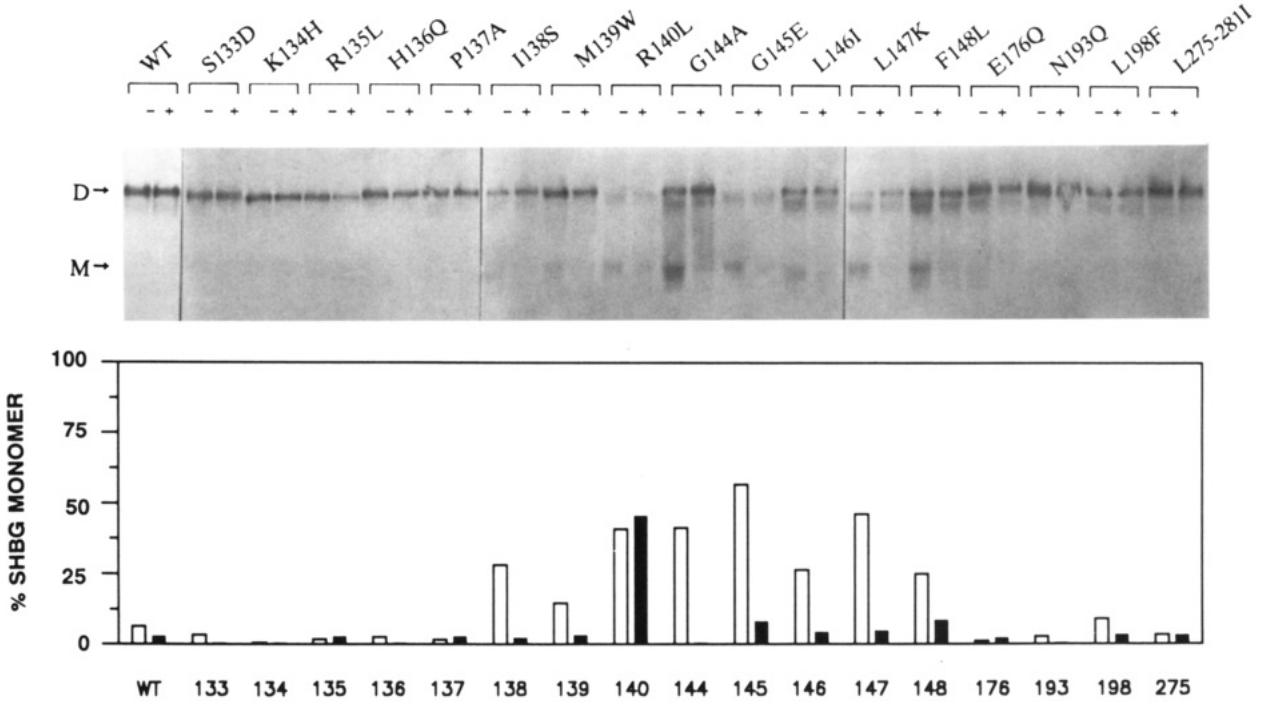

FIGURE 3: Densitometric analysis of Western blots of wild-type and mutant SHBGs in the presence (+) or absence (–) of androgen, after nondenaturing PAGE. Steroids were removed from culture media containing wild-type or mutant SHBGs by incubating the undiluted samples with DCC for 30 min. The DCC-treated culture media were then incubated in the presence or absence of 500 nM DHT for 15 min at 37 °C, followed by 45 min at room temperature before analysis by PAGE. The positions of normal dimeric (D) and monomeric (M) forms are indicated (arrows). Western blots (upper panel) of the samples incubated in the presence (closed bars) or absence (open bars) of DHT were scanned by a densitometer (lower panel), and the values are expressed as the percentage of monomeric SHBG with respect to the total immunoreactive SHBG in each lane of the Western blots.

Densitometric analyses of the Western blots provide a semiquantitative assessment of the dimer instability of various SHBG mutants in the absence of steroid ligands (Figure 3). For this purpose, a polyclonal antiserum was selected that recognizes both monomeric and dimeric forms of SHBG on a Western blot. Although we cannot be certain that these antibodies react equally with the different conformations of SHBG immobilized on the blots, the data demonstrate that the readdition of androgen largely restores dimerization of all but one of the dimer-impaired mutants (Figure 3). However, the amount of this particular mutant (R140L) in the sample analyzed was relatively low, and it has been shown to reassociate as a dimer in the presence of androgen in other experiments (data not shown). Conversely, mutants with substitutions in SHBG at residues 134–137, which show differences in their affinities for certain steroids (Table 1), dimerize normally irrespective of the presence or absence of androgen, as did wild-type SHBG and other mutants with substitutions outside of the region I138–F148 (Figure 3).

The L147K mutant was used as an example to determine whether steroid-mediated dimerization is restricted to ligands that bind to SHBG. To accomplish this, the L147K mutant was first DCC-treated to remove steroids from the binding site and then exposed to various steroid ligands at two different concentrations. As a result, DHT, T, and E_2 were all effective at restoring dimer formation, while progesterone, which is not a ligand for SHBG, was unable to enhance dimer formation of this mutant even at the 500 nM concentration (Figure 4).

Influence of Divalent Cations on Dimerization. Divalent cations were removed from culture medium samples by EDTA treatment to ascertain whether they influence dimer formation of wild-type or mutant SHBGs in the presence of DHT. This resulted in the appearance of monomers in those mutants with amino acid substitutions in the region of residues 140–148 (Figure 5), which includes the same mutants that exhibit a reduced ability to dimerize in the absence of steroid ligands (Figure 3). As in the case of wild-type SHBG, substitutions elsewhere in the protein had essentially no effect on dimerization in the absence of divalent cations (Figure 5).

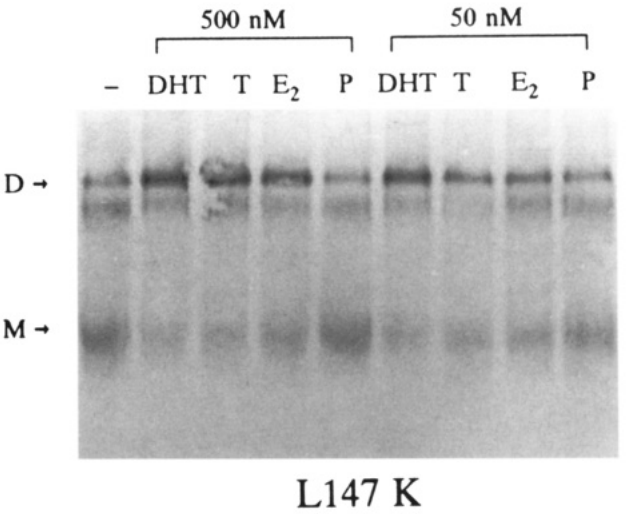

FIGURE 4: Western blot of the L147K SHBG mutant after incubation with different steroids. The DCC-treated L147K mutant was incubated alone (–) or in the presence of 5α-dihydrotestosterone (DHT), testosterone (T), estradiol (E_2), and progesterone (P) at 500 and 50 nM concentrations. The relative positions of normal dimeric (D) and monomeric (M) immunoreactive forms of SHBG are indicated (arrows).

Since the removal of steroid ligands or divalent cations destabilizes only those mutants containing amino acid substitutions at residues 140–148, both steroids and divalent cations were removed from culture media containing wild-type SHBG or the L147K mutant. This was done to determine

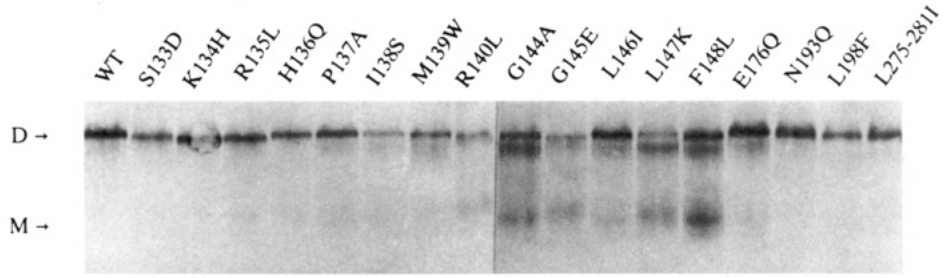

FIGURE 5: Western blot of wild-type and mutant SHBGs after treatment with EDTA. Culture medium containing each sample in the presence of 100 nM DHT was exposed to 5 mM EDTA for 15 min to remove divalent cations, prior to analysis by PAGE. The relative positions of normal dimeric (D) and monomeric (M) immunoreactive forms of SHBG are indicated (arrows).

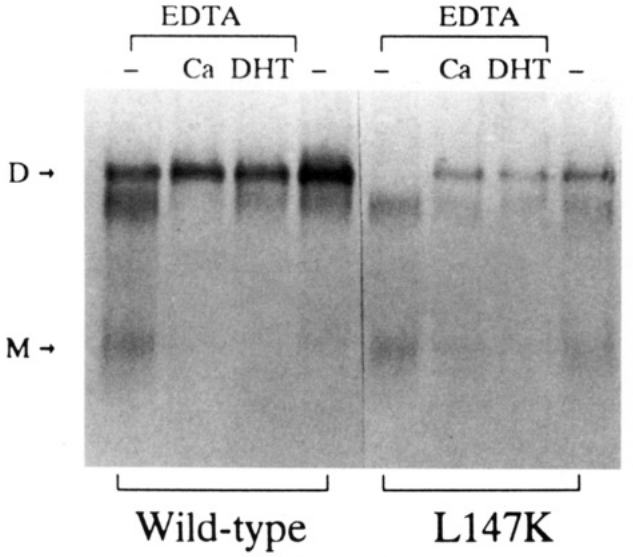

FIGURE 6: Western blot of wild-type and L147K SHBGs after the removal and readdition of both 5α-dihydrotestosterone (DHT) and $CaCl_2$ (Ca). Culture media containing wild-type or L147K SHBGs were exposed to DCC to remove steroid and then treated with 5 mM EDTA to remove divalent cations. The samples were then incubated alone (–) or in the presence of 500 nM DHT or 10 mM $CaCl_2$ for 15 min at 37 °C, followed by 45 min at room temperature before PAGE. The relative positions of normal dimeric (D) and monomeric (M) immunoreactive forms of SHBG are indicated (arrows).

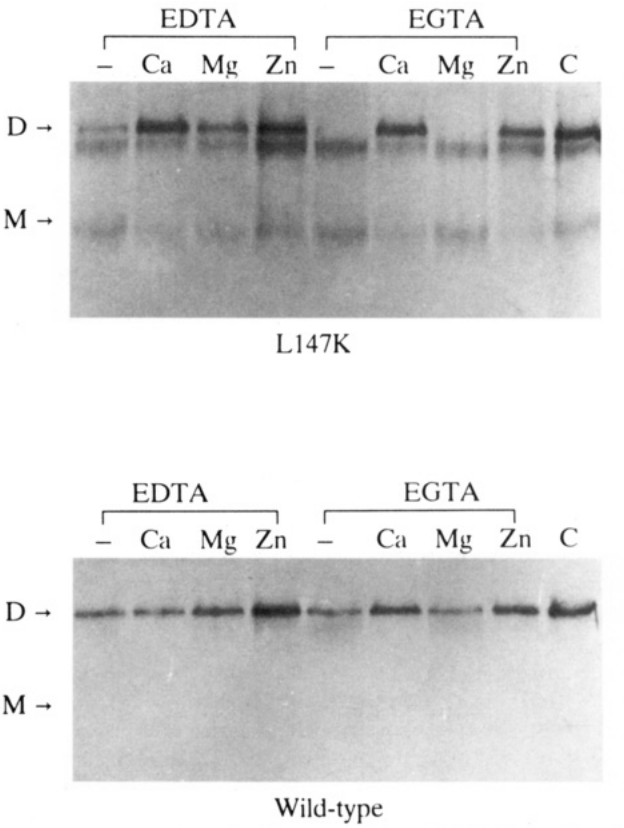

FIGURE 7: Western blot of wild-type and L147K SHBGs incubated in the presence and absence (–) of divalent cations. Culture media containing wild-type or L147K SHBGs in the presence of 100 nM DHT were treated with 5 mM EDTA or 5 mM EGTA and then incubated alone (–) or with 10 mM $CaCl_2$ (Ca), 10 mM $MgCl_2$ (Mg), or 10 mM $ZnCl_2$ (Zn), prior to analysis by PAGE. Untreated culture media containing wild-type or L147K SHBGs were analyzed as controls (C). The relative positions of normal dimeric (D) and monomeric (M) immunoreactive forms of SHBG are indicated (arrows).

whether the absence of both components would further exacerbate dimer instability. As expected, the L147K mutant completely lacks a normal dimeric phenotype under these conditions, but readdition of either DHT or calcium regenerates dimer formation of this mutant (Figure 6). It should be noted that the dimerization of wild-type SHBG is also compromised in the absence of both steroid and calcium to a limited extent, but again the readdition of either DHT or calcium can restore dimer formation. This result is also important because it demonstrates that the diffuse band, with a slightly greater mobility ($R_f = 0.26$) than the normal SHBG dimer ($R_f = 0.20$), disappears after the readdition of calcium. Therefore, this supports our assumption that it represents a dimer with altered conformation, as opposed to being a degradation product.

Although calcium restores SHBG dimer formation, magnesium is also present in the culture medium. We therefore examined whether other divalent cations could produce the same effects as calcium. To illustrate this, wild-type SHBG and the L147K mutant were treated separately with EDTA or EGTA in the presence of DHT, and various divalent cations were then added back individually. After treatment with EDTA, the L147K mutant did not completely dissociate into monomer, and Ca^{2+} or Zn^{2+}, but not Mg^{2+}, was capable of regenerating dimer formation (Figure 7). By comparison, EGTA (a specific chelator of Ca^{2+}) is much more effective than EDTA at dissociating L147K, as illustrated by the complete loss of normal dimer, and only calcium and zinc restored its dimeric state. By contrast, the normal dimeric phenotype of wild-type SHBG remained unaffected by any of the treatments, illustrating the specificity of these observations.

DISCUSSION

Although a recent study in which radical alterations were made throughout the entire sequence of rat ABP failed to locate any particular region that might represent the steroid-binding site (Joseph & Lawrence, 1993), we have found that amino acid substitutions in a region encompassing M139 of human SHBG induce subtle changes in its steroid-binding

and dimerization properties. When compared to this previous study, in which only two of eleven rat ABP mutants were secreted (Joseph & Lawrence, 1993), the changes we made in the human SHBG sequence were essentially confined to single amino acid substitutions, and this resulted in the secretion of all but two of our mutants. Human SHBG also binds sex steroids with higher affinity than rat ABP (Westphal, 1986), and this has allowed us to characterize the steroid-binding properties of our mutant proteins in much greater detail. Furthermore, recombinant proteins that are misfolded during synthesis in mammalian cells are often poorly secreted and/or undergo rapid degradation (Rose & Doms, 1988), and the overall structures of most of our SHBG mutants are probably not substantially altered because they are secreted in amounts similar to wild-type SHBG. More importantly, as in a similar study of the estrogen receptor (Fawell et al., 1990), we have also been able to discriminate between amino acids that influence either steroid binding or dimerization, and the retention of at least one of these functions further suggests that the changes we have introduced do not significantly alter the protein structure.

Substitutions of M139 in human SHBG almost invariably cause marked reductions in its affinity for androgen (Bocchinfuso et al., 1992; Sui et al., 1992). We have now identified a phylogenetically poorly conserved region immediately N-terminal to M139, where other substitutions result in alterations in steroid-binding specificity. The most notable of these are the abnormal affinities for estradiol that result from altering residues 134–138, which is interesting because sex steroid-binding proteins in subprimate species generally bind this estrogen with very low affinities (Renoir et al., 1980). These data also suggest that the unique nonplanar configuration of estradiol (Duax & Norton, 1975) positions the phenolic A ring closer to this series of residues within the human SHBG-binding site or at the entrance to it. Consistent with this hypothesis is the observation that the R135L mutant binds steroid ligands with a 3β-hydroxyl group (oriented above the plane of the molecule) with approximately 2-fold greater affinity. Mutations in this region of SHBG do not affect the binding of more planar androgens with a 3-keto group, and this also argues against major perturbations in the binding site.

Analysis of the M139W mutant has demonstrated that it binds T, 5αA, and E_2 with the same relative affinities as wild-type SHBG. However, M139W binds DHT with 5-fold weaker affinity than wild-type SHBG (Bocchinfuso et al., 1992), and its actual affinities for T, 5αA, and E_2 must also be equally reduced. By contrast, M139W shows a striking 5-fold increase in its relative affinity for 19norT, which suggests that this amino acid substitution does not affect the ability of the mutant SHBG to bind this particular steroid. Therefore, it is possible that the lack of a methyl group at C19 in 19norT tolerates the replacement of the methionine for a bulky tryptophan at this position. Unlike the configuration of the phenolic A ring of E_2, the 19norT A ring bends below the plane of the molecule, and this could also circumvent steric hindrance by the tryptophan in M139W. In addition, the M139W mutant also has an increased relative affinity for Δ5A, but to a lesser extent when compared to 19norT. Although Δ5A contains a C19 methyl group, the double bond between C5 and C6 may alter the configuration of the B ring such that this steroid ligand is not sterically hindered to the same degree as the other C19 steroids, DHT, T, and 5αA.

We suggest that residues 134–139 normally make contact with the steroid ligand and that substitutions at these positions may create local perturbations in the SHBG steroid-binding site that more readily accommodate steroids with specific A/B ring conformations or hinder their recognition. These data suggest that steroid ligands are oriented in the binding pocket such that amino acids 134–138 are situated near their A and B rings, while M139 interacts with the steroid via the B ring. This concept of the steroid-binding site contrasts with the affinity labeling of K134 using a 17β-bromoacetoxy derivative of DHT (Namkung et al., 1990). However, the conclusion that K134 interacts with the D ring of this affinity label is debatable because the 17β-bromoacetoxy group may not accurately define the amino acid closest to that locus on the steroid, which may explain why it has been reported to also interact with H235 (Khan & Rosner, 1990).

The dimerization interface of SHBG has not been examined previously at the molecular level, but amino acid substitutions within one of the most hydrophobic regions of the molecule (I138–F148) clearly impair its ability to dimerize. The removal of steroid ligand destabilizes the dimerization of these particular mutants, while readdition of steroids that bind to SHBG with high affinity promotes subunit reassociation. These observations suggest that these residues partially overlap with a region that influences steroid-binding activity. Interestingly, a similar study of the estrogen receptor has demonstrated that its steroid binding and dimerization domains overlap (Fawell et al., 1990; White et al., 1991), and it has also been shown that estrogen and androgen receptor dimerization is ligand-inducible (Kumar & Chambon, 1988; Wong et al., 1993).

Only single amino acid substitutions were made throughout the region surrounding M139. In the hydrophobic region immediately C-terminal to M139, the substitutions were largely confined to residues that are absolutely conserved between species (Hammond, 1993). This was done because it was initially anticipated that they might constitute at least part of a hydrophobic steroid-binding pocket, as was suggested by others (Danzo et al., 1991). Surprisingly, these alterations had no impact on steroid-binding activity, but all of them destabilized dimer formation, as assessed by their electrophoretic properties under nondenaturing conditions. Within this region, it is clear that effects on dimerization are not simply confined to changes in hydrophobicity. It is therefore likely that the loss of a particular local conformation and/or a hydrophobic residue may alter the nature of a hydrophobic interface essential for efficient subunit association.

In rabbit SHBG, residues 141 (leucine) and 143 (valine) differ from the isoleucine and leucine residues that are found at these positions of the protein in other species (Hammond, 1993), and we presumed they may not contribute to steroid binding. On the other hand, A142 is invariant across species, and its alteration to a charged residue (E) resulted in a complete loss of secretion, which may be attributed to destabilization of the molecule. The same argument may apply to two other mutations in this region that were designed to replace the phylogenetically conserved, hydrophobic residues at positions 144 (G→E) and 146 (L→K) with more hydrophilic residues, which also essentially eliminated secretion. These substitutions probably severely disrupt the structure of the molecule, especially as more conservative changes at these positions (G144A and L146I) result in the secretion of SHBG mutants with normal steroid-binding properties. However, these latter mutants are defective in their abilities to dimerize in the absence of steroid, which implies that G144 and L146 may participate in the formation of a discrete hydrophobic domain required for strong subunit association. It is also remarkable

that these mutations have such an impact on the ability of SHBG to dimerize because a mutant (L275–281I), in which four conserved leucines were all replaced with isoleucine, binds steroid and dimerizes appropriately. Thus, the region of alternating leucines within the C-terminal portion of the molecule, i.e., from L267 to L281, may not participate in either steroid binding or dimerization. By contrast, the region we have identified as participating in dimerization is sensitive to even minor changes in the size (G144A) or spatial isomerization (L146I) of amino acid side chains.

Partially purified SHBG rapidly and irreversibly loses steroid-binding activity, but this can be delayed if androgen and calcium are present in the storage buffer (Rosner et al., 1974). The removal of divalent cations from the culture medium destabilizes the dimerization of mutants that are also affected by the removal of steroid, and this may explain the stabilizing effects of these agents during storage. More importantly, the readdition of either calcium or androgen can independently promote dimer formation; therefore, their modes and sites of action in this regard are probably unrelated. Wild-type SHBG dimer formation is only compromised in the absence of both divalent cation and steroid and probably reflects the greater efficiency of its intact dimerization domain, when compared to the compromised state of the dimerization domains of L147K and other mutants with substitutions in this region.

We cannot be certain that Ca^{2+} is required to stabilize SHBG in biological fluids, but our results demonstrate a degree of specificity in the way SHBG associates with cations because Mg^{2+} clearly is not involved in this process. There are no obvious structural similarities between SHBG and proteins that contain well-characterized Ca^{2+}-binding domains (Trigo-Gonzalez et al., 1993; Concha et al., 1993; Babu et al., 1985), but it has been postulated that cation-binding sites are located at or near the hormone-binding domain of the estrogen receptor (Jaouen et al., 1993). Furthermore, SHBG exhibits some sequence identity with protein S (Joseph & Baker, 1992), the calcium-binding properties of which are an essential facet of its function as a cofactor in the blood coagulation pathway (Dahlbäck et al., 1990).

The correct tertiary structure of SHBG requires the formation of two intramolecular disulfide bridges, and this may explain why we have failed to observe the secretion of SHBG mutants lacking either pair of cysteines that form these bridges (unpublished data). It is possible that these bridges are necessary for appropriate folding of the molecule and that one or both of them are required to ensure the correct formation of the dimerization and/or steroid-binding domains. Since the quaternary structure of SHBG apparently can be disrupted and then regenerated by the readdition of steroid ligands, calcium, or zinc, it would appear that each SHBG subunit folds independently prior to dimerization. Whether dimerization occurs intracellularly during posttranslational processing or during secretion is not known, but given the low concentrations of intracellular Ca^{2+}, it is likely that this cation associates with the protein extracellularly after dimerization in order to stabilize it.

In conclusion, the data we have presented are consistent with the hypothesis that high-affinity steroid binding is determined by the presence of specific amino acids in the N-terminal half of SHBG (Bocchinfuso et al., 1992). More specifically, our data indicate that amino acids at positions 134–139 interact with the A/B ring conformations of steroid ligands. Furthermore, we have found that this region partially overlaps with a series of predominantly hydrophobic residues (138–148) that appear to constitute an important component of the dimerization interface between SHBG monomers, and we believe that steroid ligands reside in close proximity to this interface. We also demonstrate that divalent cations stabilize dimer formation, and we provide evidence that Ca^{2+} may be responsible for this. Since several other steroid-binding proteins form homodimers in the presence of steroid, including uteroglobin (Miele et al., 1987) and the estrogen and androgen receptors (Fawell et al., 1990; White et al., 1991; Kumar & Chambon, 1988; Wong et al., 1993), further structural analysis of SHBG may provide insight into how these proteins associate as homodimers and/or bind steroid ligands.

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