Resolution of the Steroid-Binding and Dimerization Domains of Human Sex Hormone-Binding Globulin by Expression in *Escherichia coli*†

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ABSTRACT: To determine the minimal sequence requirements for steroid binding and dimerization of human sex hormone-binding globulin (SHBG), the SHBG polypeptide and various SHBG deletion mutants were expressed as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli.* Fusion proteins containing the complete SHBG sequence, or the first 177 N-terminal residues of SHBG, bound steroids with high affinity and specificity. Further deletions from the C-terminus severely compromised steroid-binding activity, as did N-terminal deletions beyond residue 18 in the SHBG sequence. Thus, residues 18−177 in SHBG encompass a region required for its steroid-binding activity, and a disulfide bridge normally present between Cys-164 and Cys-188 in SHBG is not obviously essential for steroid binding. Most of the GST/SHBG fusion proteins undergo cleavage at 4 °C, releasing immunoreactive polypeptides that correspond approximately in size to their respective SHBG sequences. The 23-kDa immunoreactive cleavage product released from the fusion protein containing residues 1−205 in the SHBG sequence (SHBG 1−205) has a 50-fold greater steroid-binding capacity but a 7.5-fold lower affinity than its parent fusion protein. In addition, the 22-kDa immunoreactive polypeptide released from SHBG(1−194) binds steroid, and its dimerization is promoted by steroid ligands that bind SHBG with high affinity. These data suggest that the N-terminal region of SHBG dimerizes readily in the absence of GST and in doing so acquires steroid-binding sites. In conclusion, the N-terminal half of SHBG contains all the essential elements for steroid binding and dimerization, and we suggest that the C-terminal region may stabilize the overall conformation of SHBG to enhance steroid-binding affinity and/or provide some other function.

Plasma sex hormone-binding globulin (SHBG)[1] and testicular androgen-binding protein (ABP) are homodimeric glycoproteins that share the same primary structure and differ only with respect to the types of oligosaccharides attached to them (Hammond et al., 1986, 1989; Danzo & Bell, 1988; Danzo et al., 1989). The biological significance of these differences is not understood, but enzymatically deglycosylated human SHBG (Petra et al., 1992) and an unglycosylated SHBG mutant (Bocchinfuso et al., 1992a) both bind steroids normally. Affinity-labeling experiments (Grenot et al., 1992; Namkung et al., 1990) and studies of recombinant human SHBG mutants (Bocchinfuso et al., 1992b; Bocchinfuso & Hammond, 1994; Sui et al., 1992) have shown that a region encompassing and including Met-139 represents an important component of the SHBG steroid-binding site. In addition, analyses of a chimeric protein, comprising the N-terminal region (205 amino acids) of human SHBG fused to the remaining C-terminal portion of rat ABP (Bocchinfuso et al., 1992b), have indicated that amino acids responsible for the much higher steroid-binding affinity of human SHBG are probably located within the N-terminal half of this molecule. These observations prompted us to express human SHBG in *Escherichia coli* as a means of studying its functional characteristics.

Attempts to define the minimal steroid-binding domain of rat ABP by expressing deletion mutants in mammalian cells have been inconclusive (Joseph & Lawrence, 1993), and this may be explained by inappropriate folding or subunit association, which could have disrupted their production and/or secretion. To circumvent these problems, and to identify an expression system capable of synthesizing large amounts of recombinant SHBG with steroid-binding activity for physicochemical analyses, human SHBG and various SHBG deletion mutants have been produced as glutathione *S*-transferase (GST) fusion proteins in *E. coli.* These mutants were designed to lack potentially important regions of the SHBG molecule which we identified on the basis of phylogenetic comparisons of its primary structure (Hammond, 1993), available secondary structural information (Walsh et al., 1986), and complementary studies of the steroid-binding and dimerization properties of SHBG mutants expressed in mammalian cells (Bocchinfuso & Hammond,

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[1] Abbreviations: SHBG, sex hormone-binding globulin; GST, glutathione *S*-transferase; ABP, androgen-binding protein; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; IRMA, immunoradiometric assay; DHT, 5α-dihydrotestosterone; T, testosterone; E_2, 17β-estradiol; P, progesterone; DCC, dextran-coated charcoal; K_d, dissociation constant; R_f, relative mobility; cpm, counts per minute.

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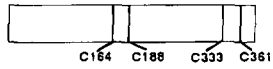
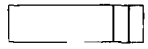
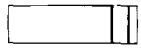
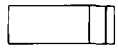
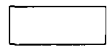
Table 1: Immunochemical and Steroid-Binding Measurements of GST/SHBG Fusion Proteins[a]

#	Regions of SHBG expressed as GST fusion proteins		GST/SHBG	BCA	IRMA	K_d	Off-Rate
	Name	Diagram	(MW x 10^{-3})	(pmol/ml)		(nM)	(%)
1.	SHBG(1-373)		67	20.0	26.0	12.5	30
2.	SHBG(1-205)		49	16.9	26.0	3.8	4
3.	SHBG(1-194)		48	26.8	23.7	3.8	4
4.	SHBG(1-177)		46	9.4	1.4	9.0	16
5.	SHBG(18-194)		46	0.2	0.1	—	—
6.	SHBG(29-194)		45	0	0.02	—	—
7.	SHBG(1-163)		44	0	0	—	—
8.	SHBG(39-194)		43	0	0	—	—
9.	SHBG(1-151)		43	0	0	—	—
10.	SHBG(1-142)		42	0	0	—	—
11.	SHBG(101-194)		37	0	0	—	—

[a] The GST/SHBG fusion proteins expressed in *E. coli* are numbered (#) 1−11 and named according to the region of the SHBG polypeptide contained within their coding sequences, with the residues of SHBG shown in parentheses. Equivalent amounts of fusion proteins were analyzed by an immunoradiometric assay (IRMA) specific for human SHBG and a steroid binding capacity assay (BCA). The apparent dissociation rate (Off-Rate) of protein-bound [^{3}H]DHT was measured by exposure to dextran-coated charcoal for increasing times at 0 °C, and dissociation constants (K_d) were measured by Scatchard analysis. The predicted molecular weights (MW) of the various fusion proteins are listed. The positions of conserved cysteines in the SHBG polypeptide are shown in the diagram of SHBG(1−373).

1994). As a result, we now demonstrate that the steroid-binding domain is confined to the N-terminal half of the protein, and that dimerization of this portion of the molecule is enhanced by the presence of steroid ligands.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors. A human SHBG cDNA in the pSELECT-1 vector (Promega) was mutated using an oligonucleotide primer (according to the protocol provided by Promega) to introduce a *Bam*HI site immediately 5′ to the codon for Leu-1 in the mature polypeptide (Hammond et al., 1987; Bocchinfuso et al., 1992b). The resulting cDNA was then excised with *Bam*HI and inserted into a *Bam*HI-digested pGEX-2T vector (Pharmacia) to create a cDNA that encodes a GST/SHBG fusion protein, designated SHBG(1−373) as shown in Table 1. Digestion of this cDNA with either *Eco*RI, *Sma*I, or *Bgl*II and *Eco*RI and religation of the remaining constructs resulted in cDNAs that encode fusion proteins containing the N-terminal 205, 194, and 177 amino acids of SHBG, respectively [see SHBG-(1−205), SHBG(1−194), and SHBG(1−177) in Table 1]. The SHBG(1−163), SHBG(1−151), and SHBG(1−142) C-terminal deletion mutants were derived from the SHBG-(1−194) construct by amplification of the SHBG coding sequence by polymerase chain reaction (PCR) using 3′ primers to introduce an *Eco*RI site immediately 3′ of the codon for residue 163, 151, or 142 in the SHBG polypeptide (see Table 1). The 5′ primer used for this purpose corresponds to nucleotides 855−871 in the pGEX-2T vector. After digestion with *Bam*HI and *Eco*RI, the PCR products were cloned into pGEX-2T and sequenced to confirm that no errors had been introduced by Taq polymerase. The N-terminal deletion mutants SHBG(18−194), SHBG(29−194), SHBG(39−194), and SHBG(101−194) were created in a similar manner except that a 3′ primer corresponding to nucleotides 1023−1005 in pGEX-2T was used with different 5′ primers to introduce a *Bam*HI site immediately 5′ to the codons for residues 18, 29, 39, or 101 in the SHBG sequence (Table 1).

Expression and Purification of Fusion Proteins. The different pGEX-2T constructs were used to transform JM107 or BL21 *E. coli* strains, and preliminary experiments indicated that expression in the JM107 strain resulted in consistently greater yields of full-length products (data not

shown). The JM107 strain was therefore used for expression of GST fusion proteins (Smith & Johnson, 1988) with the following modifications: bacterial cultures (250 mL) were grown for approximately 4 h at 37 °C (A_{600} of 0.7−1.0), cooled for 15 min at room temperature prior to addition of 0.5 mM isopropyl β-D-thiogalactoside (Promega), and further incubated for 90 min at 29 °C. The cultures were centrifuged (5000*g* for 10 min at 4 °C), and the bacterial pellets were resuspended in 50 mL of ice-cold phosphate-buffered saline (PBS). Lysozyme (Sigma) was added to a concentration of 1 mg/mL prior to incubation in an ice−water bath (0 °C) for 20 min. Triton X-100 (BDH) was then added to a final concentration of 1%, and the suspensions were incubated at 0 °C for 10 min before ultrasonic disruption. The sonicated suspensions were centrifuged for 1 h at 9000*g*, and the supernatants containing soluble proteins were passed through a 0.45 μm syringe filter (Nalgene). The filtered solutions were passed twice through a 3-mL glutathione−Sepharose column (Pharmacia). This and subsequent chromatographic procedures were performed at room temperature. After the columns were washed with PBS, the bound protein was eluted with 50 mM Tris-HCl, pH 8.0, containing 10 mM reduced glutathione. Fractions containing eluted GST fusion proteins were pooled, NaCl was added to a final concentration of 3 M, and free glutathione was removed using a 3 mL Sephadex G-25 column (Pharmacia).

Electrophoretic Analyses. The fusion proteins were analyzed under different experimental conditions by polyacrylamide gel electrophoresis (PAGE) and western blotting (Towbin et al., 1979). Samples were subjected to sodium dodecyl sulfate (SDS)−PAGE or nondenaturing PAGE (4% stacking gel and 12% or 15% resolving gel), and proteins were visualized in the gels by staining with Coomassie Brilliant Blue G (Sigma) or transferred electrophoretically to an Immobilon-P membrane (Millipore). The blots were blocked with 5% powdered milk solution prior to incubation with a rabbit antiserum against human SHBG (diluted 1/500), and immunoreactive proteins were detected using a goat anti-rabbit IgG alkaline phosphatase-labeled secondary antibody (diluted 1/5000), as described previously (Bocchinfuso et al., 1991).

Immunochemical and Steroid-Binding Analyses. Relative concentrations of the affinity-purified fusion proteins were assessed by densitometric quantification of the appropriately sized bands on Coomassie-stained SDS−PAGE gels (Table 1 and Figure 1A). This allowed us to measure an equivalent amount of each fusion protein with an immunoradiometric assay (IRMA) that is specific for human SHBG (Hammond et al., 1985). Equivalent amounts of fusion proteins were also taken for saturation analysis with 5α-dihydrotestosterone (DHT) as the ligand (Hammond & Lähteenmäki, 1983). In brief, samples were incubated with 10 nM [^{3}H]DHT (43.7 Ci/mmol, Dupont Canada) in the presence or absence of a 200-fold molar excess of nonradioactive DHT to monitor nonspecific binding. Bound and free steroids were separated using dextran-coated charcoal (DCC). The rate of [^{3}H]DHT dissociation from the various proteins was determined by exposure to DCC for increasing time periods at 0 °C, and the dissociation rate was taken into account when calculating steroid-binding capacity (Hammond & Lähteenmäki, 1983). The affinities of various fusion proteins for DHT were estimated by Scatchard analysis, and their relative binding affinities for other steroids were determined using [^{3}H]DHT as the radioactive ligand and increasing amounts of nonradioactive DHT, testosterone (T), 17β-estradiol (E_2), or progesterone (P) as competitors (Hammond & Lähteenmäki, 1983).

RESULTS

Electrophoretic Properties of Fusion Proteins. After purification by affinity chromatography, fusion proteins were immediately subjected to SDS−PAGE and Coomassie stained to determine their abundance, purity, and integrity. In most instances, the major products were of the expected molecular size (see Table 1 and Figure 1A), and their relative amounts were measured by densitometry. In some cases, additional products were present with molecular sizes that were 2−5 kDa larger than the 27-kDa GST component of the fusion proteins (Figure 1A). These could not be removed by an additional glutathione−Sepharose affinity-chromatography step and probably represent premature translation termination products. Therefore, it was important to exclude these defective products when calculating the relative amounts of intact fusion proteins by densitometry for further analysis. These samples were also analyzed by SDS−PAGE and western blotting, and some additional immunoreactive bands could be detected in most lanes due to the sensitivity of this technique (Figure 1B). The number of additional immunoreactive bands varied considerably between different fusion proteins; this was most pronounced in the case of the fusion protein containing the entire SHBG sequence (Figure 1B, lane 1).

Immunochemical and Steroid-Binding Properties of Fusion Proteins. As observed with SHBG in plasma (Hammond et al., 1985), there was a good correspondence between measurements of the steroid-binding capacity and immunoreactivity of fusion proteins that contain at least 194 N-terminal amino acids of SHBG (Table 1). Removal of 17 additional C-terminal amino acids to yield SHBG(1−177) resulted in the loss of Cys-188 that normally forms a disulfide bridge with Cys-164 (Walsh et al., 1986). The steroid-binding affinity of this mutant was only about 2.5-fold lower than SHBG(1−205) or SHBG(1−194), despite a 7-fold discrepancy between its steroid-binding capacity and concentration measured by IRMA (Table 1). This relative decrease in immunoreactivity is probably due to the disruption of the conformation-dependent epitope recognized by a monoclonal antibody used in the IRMA (Hammond & Robinson, 1984) and which is known to comprise at least residues 136−138 in human SHBG (Bocchinfuso & Hammond, 1994). Any further deletions from the C-terminus, i.e., SHBG(1−163), SHBG(1−151) and SHBG(1−142), resulted in a complete loss of both steroid-binding activity and immunoreactivity (Table 1). By contrast, deletion of the first 17 N-terminal amino acids, i.e., SHBG(18−194), reduced the steroid-binding capacity and immunoreactivity equally, while further N-terminal deletions of this region, i.e., SHBG(29−194), SHBG(39−194), and SHBG (101−194), abolished both steroid binding and immunoreactivity (Table 1). When *E. coli*-expressed GST was examined as a control in these assays, it was not immunoreactive and lacked steroid-binding activity.

Fusion proteins exhibiting appreciable levels of steroid binding were examined further by Scatchard analysis, as well as by measurements of steroid dissociation rate and steroid-

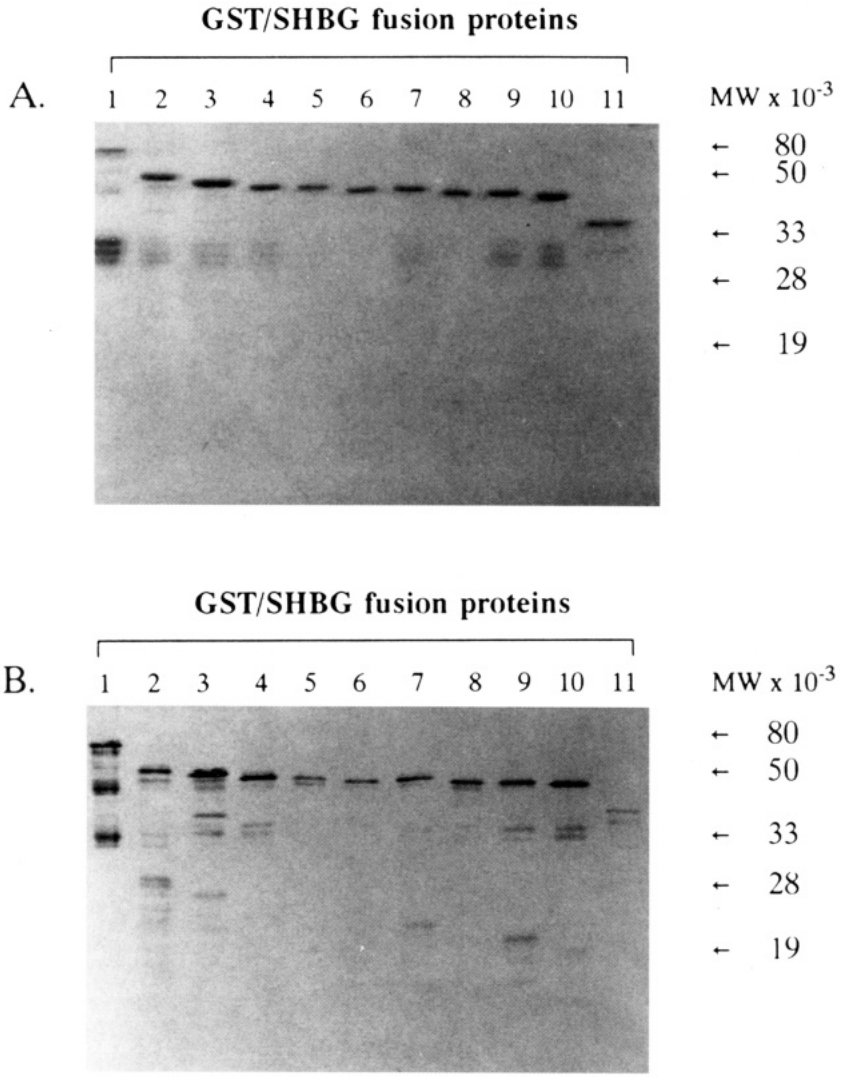


FIGURE 1: Electrophoretic properties of GST/SHBG fusion proteins. Human SHBG and various SHBG deletion mutants expressed in *E. coli* as GST fusion proteins (1−11 as listed in Table 1) were subjected to SDS−PAGE and analyzed by Coomassie staining (A) or western blotting with a rabbit antiserum specific for human SHBG (B). The molecular sizes of the major Coomassie-stained products correspond to those predicted for individual fusion proteins (see Table 1).

binding specificity. As expected, there was a direct relationship between the equilibrium dissociation constants and the apparent rates of steroid dissociation from the various deletion mutants (Table 1). Surprisingly, the SHBG(1−194) and SHBG(1−205) deletion mutants had a 3-fold greater affinity for DHT than SHBG(1−373). This variation in binding affinity may be related to a difference in the stability (See Figure 1B, lane 1), folding, and/or ability of the SHBG-(1−373) molecules to dimerize. The relatively low steroid-binding affinity of SHBG(1−177) may also be explained in the same way, especially as its immunoactivity is greatly reduced. Although SHBG(18−194) had a limited but detectable steroid-binding capacity (Table 1), further deletions from the N-terminus of SHBG virtually eliminated steroid binding and immunoreactivity. Thus, the minimal steroid-binding segment we have been able to delineate comprises the region between Leu-18 and Ile-177 in the SHBG polypeptide.

The two deletion mutants with the highest affinity for DHT, i.e., SHBG(1−205) and SHBG(1−194), bound other steroid ligands with essentially the same relative affinity as SHBG(1−373) and a recombinant human SHBG control (Figure 2).

Effect of Storage on the Integrity and Steroid-Binding Activity of Fusion Proteins. During the process of analyzing these proteins, the steroid-binding capacity of several preparations increased by as much as 2-fold over a period of 4−5 weeks when stored at 4 °C. Therefore, samples stored under these conditions were re-analyzed by SDS−PAGE and western blotting. In most cases, the appearance of a major protein of 29 kDa was obvious by Coomassie staining (Figure 3A). This protein is not recognized by antibodies against SHBG, and it most probably corresponds to the N-terminal GST component (27 kDa) of the fusion proteins plus a ~2-kDa region of the adjacent SHBG sequences (Figure 3A). This is supported by the observation that immunoreactive proteins of the size expected for the remaining SHBG portions had accumulated in these samples (Figure 3B). In some cases, these immunoreactive products were heterogeneous with respect to their size (Figure 3B, lanes 6, 7, 9, and 10), and the sizes of the major immunoreactive products released from SHBG(1−373) indicate that it must undergo additional cleavage (Figure 3B, lane 1) to yield two immunoreactive polypeptides that are at least 10 kDa smaller than the size of the entire SHBG sequence (41 kDa). By contrast, the major immunoreactive proteins

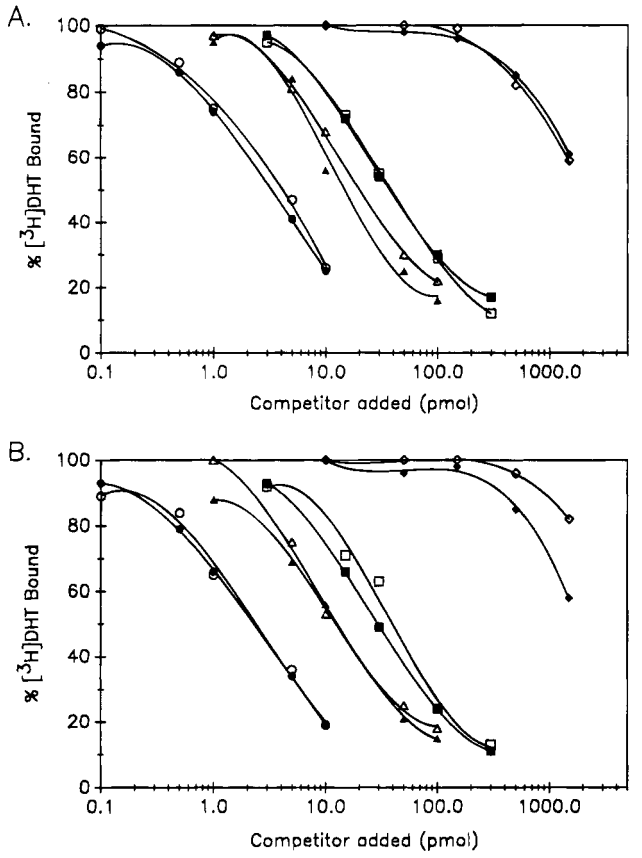


FIGURE 2: Affinities of two GST/SHBG fusion proteins for various steroid ligands relative to 5α-dihydrotestosterone (DHT). Competition curves for (A) SHBG(1−205) and (B) SHBG(1−194) are shown as closed symbols and are compared with curves for the GST fusion protein containing either (A) the entire SHBG sequence SHBG(1−373) or (B) recombinant SHBG expressed in Chinese hamster ovary cells, which are shown as open symbols. For these studies [^{3}H]DHT was used as radioactive ligand, and increasing amounts of nonradioactive DHT (○, ●), T (△, ▲), E_2 (□, ■), and P (◇, ◆) were used as competitors.

released from SHBG(1−205), SHBG(1−194), and SHBG-(1−177) appeared to be relatively stable (Figure 3B, lanes 2, 3, and 4, respectively), and their steroid-binding capacity increased during storage at 4 °C.

On the basis of these observations, one of these deletion mutants, SHBG(1−205), was examined further to determine whether the steroid-binding activity was associated with its released 23-kDa immunoreactive polypeptide or the intact fusion protein (Figure 3B, lane 2). To accomplish this, SHBG(1−205) was produced and stored at −80 °C until its composition was compared with a sample of the same protein stored at 4 °C. Western blotting demonstrated that the majority of the immunoreactivity in the sample stored at −80 °C is associated with the intact fusion protein (Figure 4A). More importantly, the 23-kDa polypeptide was the predominant immunoreactive material in the sample kept at 4 °C, but was undetectable in the sample stored at −80 °C, even when analyzed at the highest concentrations (Figure 4A).

When these proteins were examined for their ability to bind steroid, with respect to their immunoreactivity (see Figure 4A, and note the similarity in immunostaining obtained for the 1:50-diluted sample stored at 4 °C and the 1:100-diluted sample stored at −80 °C), the 23-kDa polypeptide had a 50-fold greater steroid-binding capacity than the sample stored at −80 °C (Figure 4B). Nevertheless, high concentrations (1:10 dilution) of intact fusion protein stored at −80 °C exhibited steroid-binding activity (Figure 4B), despite the absence of the 23-kDa immunoreactive polypeptide in this sample (Figure 4A). Therefore, the steroid-binding affinities of these two samples were determined by Scatchard analysis (Figure 5), which demonstrated that the 23-kDa cleavage product had a 7.5-fold lower affinity for DHT than the intact SHBG(1−205).

Dimerization and Involvement of Steroid Ligands. Dimerization has been considered a prerequisite for the formation of the SHBG steroid-binding site (Casali et al., 1990), and we have previously shown that steroid ligands can promote dimerization (Bocchinfuso & Hammond, 1994). Therefore, it was important to determine whether the immunoreactive products released from their GST fusion proteins could associate as dimers and whether dimerization could be promoted by the presence of steroid. To accomplish this, the SHBG(1−194) deletion mutant, which had been stored at 4 °C for several weeks, was applied to a second glutathione−Sepharose column. As expected, the flow-through fraction was highly enriched for a 22-kDa immunoreactive polypeptide, as demonstrated by SDS−PAGE and western blotting (not shown), and this material was then subjected to nondenaturing PAGE in gels that either lacked or contained various steroids. In the absence of steroid, most of the 22-kDa immunoreactive polypeptide migrated with a relative mobility (R_f) of 0.6, while its mobility decreased considerably (R_f = 0.3) in gels containing 100 μM DHT (Figure 6). Furthermore, when lower concentrations (350 nM) of testosterone and estradiol were included in the gels, the mobility of about half the immunoreactive protein in each sample was reduced. Progesterone interacts poorly with SHBG (Figure 2) and was unable to induce this change in electrophoretic mobility (Figure 6), which we have previously shown to be characteristic of dimer formation (Bocchinfuso & Hammond, 1994). These data clearly demonstrate that dimerization of the 22-kDa immunoreactive polypeptide derived from SHBG(1−194) is specifically enhanced by the presence of steroid ligands.

DISCUSSION

The pGEX-2T constructs for producing GST/SHBG fusion proteins in *E. coli* include a thrombin recognition/cleavage site between the GST and SHBG coding sequences and can be used to remove the GST portion of the molecule. However, our initial experiments indicated that the fusion proteins containing the entire SHBG sequence, and many of the SHBG deletion mutants, had steroid-binding capacities that closely resembled their concentrations determined by immunoassay. Consequently, it was not necessary to release the SHBG portions of these fusion proteins by thrombin digestion to demonstrate that the requirements for high-affinity binding of specific ligands are located within the N-terminal half of SHBG. These data confirm our previous assumptions about the location of the steroid-binding domain that were based on analyses of a human SHBG/rat ABP chimera (Bocchinfuso et al., 1992b) and a series of SHBG mutants with amino acid substitutions in the region of Met-139 (Bocchinfuso & Hammond, 1994).

Many of the deletion mutants we have expressed in *E. coli* bind steroids with high affinity. These findings contradict the notion that the entire molecule is necessary for

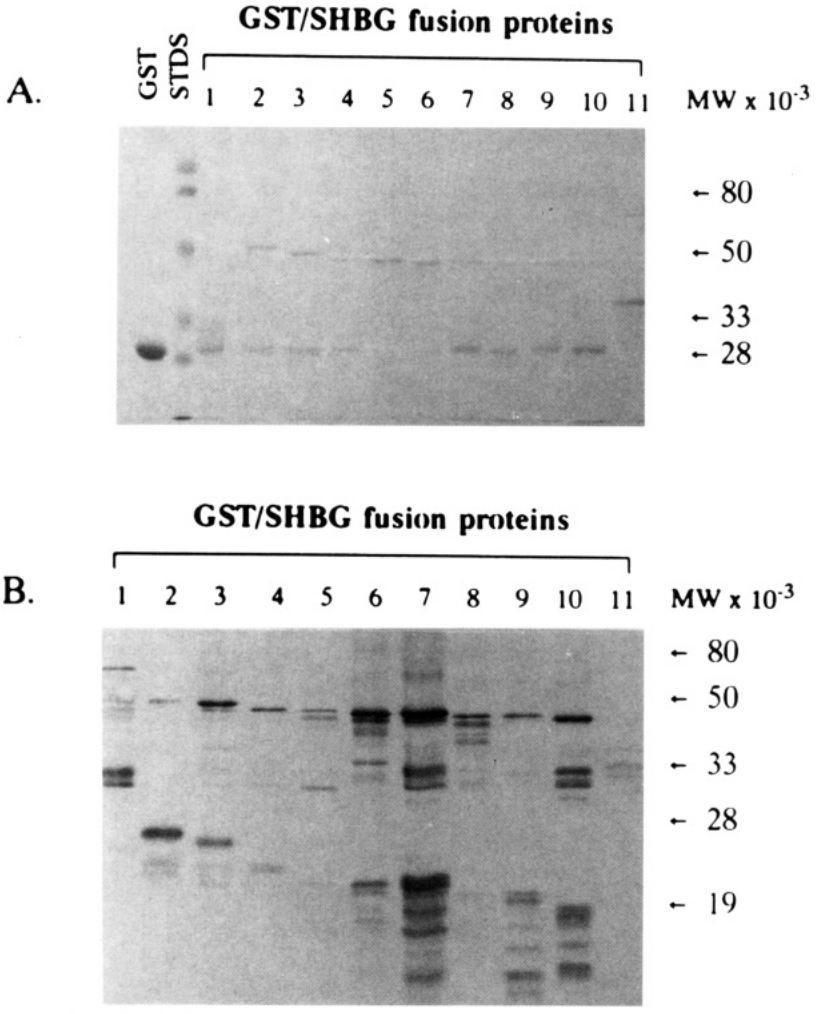


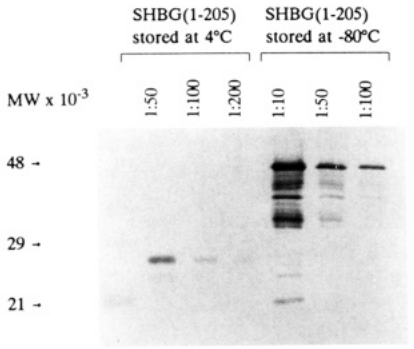
FIGURE 3: Electrophoretic properties of GST/SHBG fusion proteins showing cleavage products that accumulate during storage at 4 °C. Five weeks after their expression in *E. coli* and purification by glutathione−Sepharose affinity chromatography, samples of fusion proteins (1−11 as listed in Table 1) were analyzed by SDS−PAGE. The protein composition was determined by staining gels with Coomassie Blue (A), and immunoreactive proteins were detected by western blotting (B). In panel A, a GST standard was included as a control together with prestained molecular size markers adjacent to the lane containing fusion protein 1.

the formation of the steroid-binding site (Joseph & Lawrence, 1993) and suggest that SHBG may have defined functional domains. This is an important finding because the SHBG/ABP gene is known to produce alternatively spliced transcripts in several species, some of which could encode SHBG-related peptides that would be truncated at the C-terminus and lack a signal polypeptide required for secretion (Hammond et al., 1989; Joseph et al., 1991). The proteins encoded by these alternatively spliced transcripts have not been identified, but our current results indicate that they have the potential to bind steroids with high affinity.

Although the functional significance of the C-terminal half of SHBG remains obscure, it may influence the conformation of the molecule and provide additional structural elements that stabilize its high-affinity steroid-binding site. This concept is supported by the fact that the 23-kDa polypeptide released from SHBG(1−205) had a lower steroid-binding affinity than the intact parent molecule, which suggests that the GST sequence at the N-terminus of this molecule influences the conformation of its steroid-binding domain and enhances its affinity for ligand. In addition, the steroid-binding affinities of both the SHBG(1−205) and the SHBG-(1−194) deletion mutant are also lower than that of plasma SHBG or recombinant SHBG produced in mammalian cells (Bocchinfuso et al., 1991), which also suggests a stabilizing role for the C-terminal portion of SHBG. However, both of these deletion mutants unexpectedly bound steroid with higher affinity than the fusion protein containing the entire SHBG sequence, but this may be attributed to additional cleavage of SHBG(1−373). It is also possible that the C-terminal half of SHBG may provide some other biological function, especially as this portion of the molecule contains a consensus site for *N*-glycosylation that is invariably conserved across several mammalian species (Hammond, 1993). Human SHBG also exhibits sequence similarity with proteins that have other specialized functions, such as protein S, merosin, and laminin A (Joseph & Baker, 1992), which interact with cell membrane-binding sites (Schwalbe et al., 1990; Gehlsen et al., 1992; Engvall et al., 1992). This region of the molecule may therefore influence interactions with specific binding sites for SHBG identified within the plasma membranes of certain cell types (Strel'chyonok et al., 1984; Hryb et al., 1989; Porto et al., 1992).

Another unexpected feature of almost all the GST/SHBG fusion proteins we have produced is that they undergo cleavage during prolonged storage at 4 °C, resulting in the release of immunoreactive SHBG polypeptides. This phenomenon is unusual, and we do not believe that it can simply be attributed to the presence of a contaminating bacterial protease for the following reasons: it is not related to the

A.



B.

Dilutions	[^{3}H] DHT Bound (cpm)	
	SHBG(1-205) stored at 4°C	SHBG(1-205) stored at -80°C
1:10	—	3790
1:50	5624	454
1:100	3407	106
1:200	1809	—

FIGURE 4: Western blot and steroid-binding capacity of SHBG-(1−205) stored at −80 versus 4 °C. (A) Western blot of newly prepared SHBG(1−205) stored at −80 °C (diluted 1:10, 1:50, and 1:100) and a sample of SHBG(1−205) stored at 4 °C for 18 weeks and composed predominantly of the 23-kDa immunoreactive cleavage product (diluted 1:50, 1:100, and 1:200). (B) The steroid-binding capacities in the same dilutions of samples are expressed as the amount (cpm) of [^{3}H]DHT bound per 100 μL of sample at each dilution.

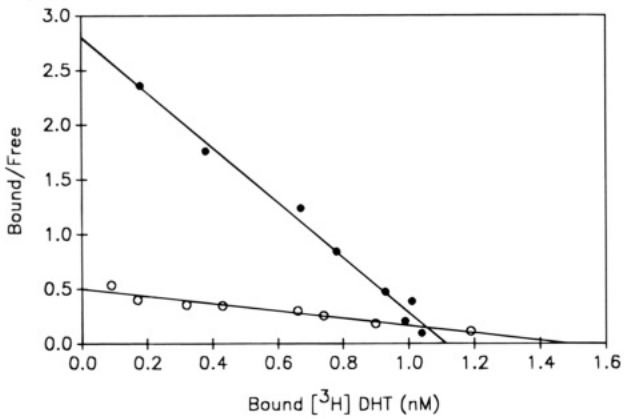


FIGURE 5: Scatchard analysis of SHBG(1−205) stored at −80 versus 4 °C. Dissociation constants (K_d) were measured at 0 °C using [^{3}H]DHT as radioactive ligand: SHBG(1−205) fusion protein stored at −80 °C, K_d = 0.4 nM (●); the 23-kDa immunoreactive cleavage product released from SHBG(1−205) after storage at 4 °C shown in Figure 4, K_d = 3.0 nM (○).

bacterial strain or pGEX vector used for expression; it is not eliminated by repurification of the intact fusion proteins; its efficiency and specificity vary between the different GST/SHBG fusion proteins, and other fusion proteins we have produced in this same way are all remarkably resistant to proteolytic degradation. There is no indication in the literature to believe that this activity has any biological significance, but the N-terminus of SHBG has been reported to be degraded proteolytically during purification or storage in serum (Walsh et al., 1986). Nevertheless, with regard to

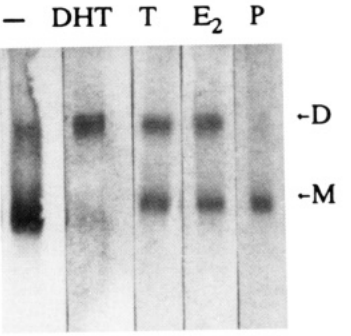


FIGURE 6: Western blot of the 22-kDa immunoreactive protein released from SHBG(1−194) and subjected to electrophoresis in the presence or absence of various steroids. Steroids were first removed from the 22-kDa immunoreactive protein released from SHBG(1−194) by exposure to dextran-coated charcoal (DCC) for 20 min. The DCC-treated protein was then incubated for 1 h at room temperature either alone (−) or in the presence of DHT, testosterone (T), 17β-estradiol (E_2), or progesterone (P) before analysis by nondenaturing PAGE in gels lacking steroid (−) or containing DHT, T, E_2, or P. The positions of dimeric (D) and monomeric (M) forms are indicated (arrows).

our immediate objectives, this cleavage activity was useful because it allowed us to conveniently separate the GST and SHBG components of some of our fusion proteins without resorting to the use of thrombin, which in our hands resulted in relatively low yields of SHBG polypeptides.

Surprisingly, the steroid-binding capacity of some of the GST/SHBG fusion proteins increased during storage at 4 °C. Since homodimerization of SHBG is thought to be necessary for the formation of a single steroid-binding site (Hammond, 1993), this increase in steroid-binding capacity may be explained by more efficient dimerization of the released SHBG polypeptides when compared to their intact fusion proteins. Indeed, we have also been able to demonstrate that the 22-kDa polypeptide released from SHBG(1−194) can be induced to dimerize in the presence of specific ligands that bind to SHBG with high affinity. These data complement previous studies where steroid was found to contribute to the energy of dimerization (Casali et al., 1990), as well as more recent data showing that a region of SHBG between residues 134 and 148 plays a role in both steroid-binding and dimerization, and that these functional domains partially overlap (Bocchinfuso & Hammond, 1994).

In summary, we have expressed a series of recombinant human SHBG deletion mutants in *E. coli*. Immunochemical and physicochemical analyses of these mutants have allowed us to demonstrate that the N-terminal half of SHBG contains the elements necessary for high-affinity steroid binding and dimerization, and that these two processes are probably interdependent. In addition, our data suggest that SHBG may have defined functional domains, and some of our deletion mutants undergo spontaneous cleavage that releases an immunoreactive steroid-binding component. It is therefore anticipated that this expression system may be exploited to produce recombinant forms of SHBG which could help define other functional properties of the protein, including its interaction with plasma membrane-binding sites. The deletion mutants may also be particularly useful for further structural analyses of the steroid-binding/dimerization domains of SHBG using nuclear magnetic resonance or X-ray diffraction techniques.

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