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Structure/function Analyses Of Sex Hormone-binding Globulin

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STRUCTURE/FUNCTION ANALYSES OF SEX HORMONE-BINDING GLOBULIN

by

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ABSTRACT

Sex hormone-binding globulin (SHBG) is a homodimeric glycoprotein that transports sex steroids in the blood and extravascular fluids, such as seminiferous tubule fluid, where it is often referred to as androgen-binding protein (ABP). A cDNA for the human SHBG precursor polypeptide was expressed in mammalian cell lines. Recombinant human SHBG bound steroids with the same affinity and specificity as natural SHBG, and was immunologically indistinguishable from its natural counterpart. Site-directed mutagenesis of the SHBG cDNA was used to introduce amino acid substitutions that selectively removed glycosylation sites from SHBG. Analysis of these glycosylation mutants demonstrated that carbohydrates are not involved in steroid binding, but the lack of both *N*-linked oligosaccharides reduced the level of SHBG secretion and/or production from CHO cells. In addition, the subunit size heterogeneity associated with SHBG is due to the differential utilization of the two consensus sites for *N*-glycosylation, and that subunits that comprise a given dimer are glycosylated and processed in the same way. An additional *N*-glycosylation site associated with an electrophoretic variant of SHBG was shown to be utilized and found not to affect steroid binding. Analyses of human SHBG/rat ABP chimeras and human SHBG *C*-terminal truncation mutants expressed in *E. coli* revealed that the steroid-binding domain is located within the *N*-terminal 205 amino acids of human SHBG. Specific amino acid substitutions in human SHBG produced mutants with altered steroid-binding specificity and demonstrated that Lys^{134}-Met^{139} interact with the A/B ring structures of steroids, and residues in a more *N*-terminal location may also contact steroid ligands. Human SHBG mutants with substitutions at residues Ile^{138}-Phe^{148} are defective in their ability to dimerize, especially in the absence of steroid and/or divalent cations, but readdition of these agents restores dimer formation. These data have led us to conclude that SHBG is a modular protein comprising an *N*-terminal steroid-binding and dimerization domain, and a *C*-terminal domain(s), which contains a phylogenetically conserved *N*-glycosylation site that may be required for other activities, such as recognition by a plasma membrane receptor.

In memory of my grandfather,

Albert Saldarelli

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ABP	androgen-binding protein
bp	base pair(s)
BSA	bovine serum albumin
°C	degrees Celsius
cAMP	adenosine 3',5'-cyclic monophosphate
CBG	corticosteroid-binding globulin
cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
Ci	Curie(s)
Con A	concanavalin A
cpm	counts per minute
Da	Dalton(s)
dATP	2'-deoxyadenosine 5'-triphosphate
DCC	dextran-coated charcoal
dCTP	2'-deoxycytidine 5'-triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis (ß-aminoethyl ether)-tetraacetic acid
FBS	fetal bovine serum
FPLC	fast protein liquid chromatography
g	gravity
GST	glutathione S-transferase
h	hour(s)
IRMA	immunoradiometric assay
kb	kilobase(s)
k_d	dissociation constant

M	molar
MEM	α-minimal essential medium
min	minute(s)
mRNA	messenger ribonucleic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RIA	radioimmunoassay
RNA	ribonucleic acid
SD	standard deviation(s)
SDS	sodium dodecylsulphate
SHBG	sex hormone-binding globulin
SSC	salt sodium citrate
SSPE	salt sodium phosphate EDTA
Taq	*Thermus aquaticus*
TBS	tris-buffered saline
Tris	tris (hydroxymethyl) aminomethane
vol	volume(s)

CHAPTER 1

INTRODUCTION

1.1 Steroid Hormone Action

Steroid hormones are synthesized by endocrine glands and secreted into the bloodstream for transportation to their target cells where they regulate gene expression. A wealth of information has accumulated concerning the 'receptor-mediated action' of steroids once entering the cell (Tsai and O'Malley, 1994). Generally, steroid receptors undergo a conformational change upon the binding of steroid ligands in the cytoplasm and this triggers the dissociation of their associated heat-shock proteins and permits possible receptor phosphorylation. After translocation of the steroid/receptor complex into the nucleus, receptors can homodimerize and bind to their cognate hormone response elements that are most often located at the 5' flanking region of target genes, where they can become further phosphorylated. At this point, receptors have the capacity to interact with other components of the transcriptional apparatus to induce or repress the expression of specific genes. Although recent data have demonstrated that in some cases, receptors can be 'activated' in a ligand-independent manner, the steroid ligand is still the major initiator of events needed to affect a change in gene transcription (Tsai and O'Malley, 1994).

1.2 Steroids in the Bloodstream

The bioavailability of steroids to responsive tissues, and their mode of entry into cells are important factors to consider when discussing receptor occupancy and activation. Steroids circulate in the blood bound to albumin, which is a high capacity, low affinity binder ($k_d = 10^{-5}$-10^{-6} M), and two high affinity ($k_d = 10^{-9}$-10^{-10} M) steroid-binding proteins, which are both characterized by a single, highly specific steroid-binding site (Westphal, 1986), namely sex hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG). Although only 1-5% of plasma steroid hormones circulate in an unbound or 'free' form (Dunn *et al.*, 1981), classical dogma states that it is only this free steroid fraction that enters target cells by passive diffusion and initiates a biological response (Siiteri *et al.*, 1982). Albumin-bound steroids have also been postulated to supplement this pool of free hormone because of the rapid dissociation of steroids from its low affinity binding sites (Siiteri *et al.*,

1982; Pardridge, 1981). Most importantly, however, variations in plasma SHBG and CBG levels greatly influence the distribution and amount of free steroid in the blood (Dunn *et al.*, 1981), and consequently control the availability of steroid to target cells (Siiteri *et al.*, 1982).

1.3 Sex Steroids and SHBG

1.3.1 Modulation of Sex Steroid Bioavailability

Plasma SHBG is the major transport protein for androgens and it binds 5α-dihydrotestosterone (DHT) with the highest affinity (k_d=5 x 10^{-10} M). However, in blood, SHBG is predominantly occupied by testosterone (T) and androst-5-ene-3ß,17ß-diol and bind both steroids with 5-fold lower affinity when compared to DHT (Westphal, 1986; Dunn *et al.*, 1981). Plasma SHBG is also capable of interacting with 17ß-estradiol (E_2) with 20-fold lower affinity relative to DHT. Therefore, SHBG influences the bioavailability of the sex steroids and some of their precursors and active metabolites by reducing the metabolic clearance rate of these steroids, which is proportional to their binding affinity for SHBG (Siiteri *et al.*, 1982). Therefore, as plasma SHBG levels decrease, the proportion of free T and E_2 in the blood increases. As a consequence, the levels of circulating SHBG will also determine the absolute concentration of sex steroids and their distribution in the blood (Siiteri *et al.*, 1982; Anderson, 1974).

Plasma SHBG is predominantly of hepatic origin, and its levels are influenced by various physiological conditions (see 1.5.1). Although, the concentration of plasma SHBG is generally two-fold greater in women than in men (Westphal, 1986; Anderson, 1974; Hammond *et al.*, 1985; Dunn *et al.*, 1981), it is evident that the level of plasma SHBG in individuals is genetically predetermined (Hammond *et al.*, 1994). In one study, the mean concentration of plasma SHBG was 23 ± 9 nM (range 11-44 nM) in men and 53 ± 24 nM (range 18-110 nM) in women, and increased 8-fold to 402 ± 172 nM (range 247-668 nM) during pregnancy, as measured by an immunoradiometric assay (IRMA) (Hammond *et al.*, 1985). Approximately 44% and 82% of circulating SHBG is unoccupied in men and

women, respectively (Dunn *et al.*, 1981) (Table 1.1). This implies that SHBG in women has a greater capacity to bind androgens, and possibly functions to prevent unwanted masculinization of sex steroid-responsive tissues.

The gene encoding SHBG (*Shbg*) is also expressed in Sertoli cells (see 1.5.2) and the brain (see 1.5.3), and this may provide a mechanism for controlling the availability of sex steroids to cells that are separated from the vasculature by a blood barrier. The protein produced in the Sertoli cells is often referred to as testicular androgen-binding protein (ABP) because it transports testosterone in the seminiferous tubule fluid and helps to maintain a high androgenic environment for the developing spermatozoa (Tindall and Means, 1980).

The pathophysiological effects of altered SHBG levels in individuals have been examined (Siiteri *et al.*, 1982; Anderson, 1974). In brief, some of the factors that increase plasma SHBG levels include, hyperthyroidism, pregnancy, anorexia nervosa and exogenous estrogen administration (Rosner, 1992; Rosner, 1990). Conversely, obesity, hyperinsulinemia, hyperandrogenism, hyperprolactinemia, increased growth hormone and synthetic progestins reduce plasma SHBG levels (Rosner, 1992; Rosner, 1990). In this regard, conditions of excessive androgen action, such as hirsutism and acne, are strongly associated with reduced levels of circulating SHBG, which lead to a higher percentage of free androgen in the blood (Anderson, 1974). Endometrial cancer is a disease of excessive estrogen action and occurs most frequently in post-menopausal obese women (Nisker *et al.*, 1980; Siiteri *et al.*, 1982). The reduction in plasma SHBG in these women coupled with the fact that their peripheral production of E_2 is elevated, results in an increased percentage of free E_2 in the bloodstream (Nisker *et al.*, 1980; Siiteri *et al.*, 1982). In summary, suppressed levels of SHBG augment both estrogen and androgen bioavailability, and may contribute to the abnormal growth and differentiation of target tissues.

1.3.2 Targeted Delivery of Sex Steroids

The hypothesis that only the free steroid fraction in the blood is biologically

Table 1.1 Plasma concentration and distribution of sex steroids and human SHBG. The percentage of the total amount of each steroid either bound to SHBG or 'free' in plasma is indicated and the percentage of unoccupied SHBG is also shown. These data are derived from Hammond *et al.*, 1985 and Dunn *et al.*, 1981.

	TOTAL (nM)	UNBOUND (%)	SHBG-BOUND (%)
MEN			
5α-Dihydrotestosterone	1.7	0.9	60.0
Testosterone	23.0	2.2	44.0
17ß-Estradiol	0.1	2.3	20.0
SHBG	24.0	44.0	-
WOMEN			
5α-Dihydrotestosterone	0.7	0.5	78.0
Testosterone	1.3	1.4	66.0
17ß-Estradiol	0.3	1.8	37.0
SHBG	53.0	82.0	-
PREGNANCY			
5α-Dihydrotestosterone	0.9	0.1	98.0
Testosterone	4.7	0.2	95.0
17ß-Estradiol	55.0	0.5	88.0
SHBG	402.0	66.0	-

active may be true for many sex steroid-dependent tissues in intimate contact with plasma, such as the endometrium. However, tissues such as the prostate, breast, and epididymis, which are less vascularized, may require more active mechanisms for internalizing specific hormones over and above that achieved by passive diffusion (Hammond, 1993; Siiteri, 1986). There is evidence that SHBG may directly participate in the delivery of steroids to these tissues *via* interaction with plasma membrane receptors (see 1.10).

1.4 The Gene Encoding SHBG

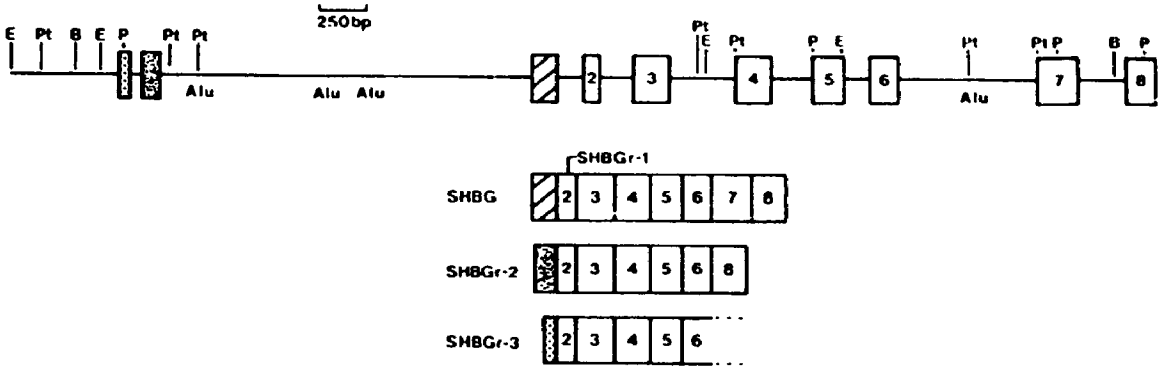
1.4.1 Chromosomal Localization

Human *Shbg* is located on chromosome 17(p12-13) in a region that contains the p53 tumour suppressor gene (Bérubé *et al.*, 1990). Specific chromosomal abnormalities have not been associated with diseases in which SHBG levels are low, but the 17p12 region is considered a fragile site within the human genome and a possible 'hot spot' for genetic recombination, gene amplification, and the integration of foreign genomes (Hori *et al.*, 1988). In addition, this fragile site appears to be located in an AT-rich region (Schmid *et al.*, 1987) and human *Shbg* contains multiple *Alu* sequences with AT-rich flanking regions (Hammond *et al.*, 1989). Allelic deletions of this region of chromosome 17(p12→p13) are frequently found in a variety of cancers, that are linked to deletions or mutations of the p53 gene (Baker *et al.*, 1989). Therefore, *Shbg* could be a useful marker for tumour-associated rearrangements or deletions of this portion of the human genome.

1.4.2 *Shbg* Coding Region

The coding region for SHBG is distributed over 8 exons that span approximately 3 kilobases (kb) of genomic DNA (Hammond *et al.*, 1989) (Figure 1.1). The gene for SHBG is expressed predominantly in hepatocytes (Khan *et al.*, 1981) and Sertoli cells (Hagenas *et al.*, 1975) yielding a 1.6 kb mRNA which encodes an open reading frame for 402 amino acids (Hammond *et al.*, 1989; Hammond *et al.*, 1987). The primary structure of human SHBG was first resolved by direct sequence analysis of the purified protein from the blood (Walsh *et al.*, 1986). Shortly afterwards, cDNA

Figure 1.1 Organization of the human SHBG gene, and exon composition of SHBG and its related (SHBGr) transcripts. Exons are represented by numbered *boxes* and the unique 5' exons of SHBG (*hatched*), SHBGr-2 (*shaded*), and SHBGr-3 (*stippled*) cDNAs are indicated. The sequence inversion of SHBGr-3 is indicated by the *dashed lines*. The positions of *Alu* sequences are shown *below* the gene map. The diagram is derived from Hammond *et al.*, 1989.


E Pt B E P Pt Pt
250bp
Alu
Alu Alu
Pt E Pt
P E
Pt
Pt P
B P
2 3 4 5 6 7 8
Alu
SHBGr-1
SHBG
SHBGr-2
SHBGr-3

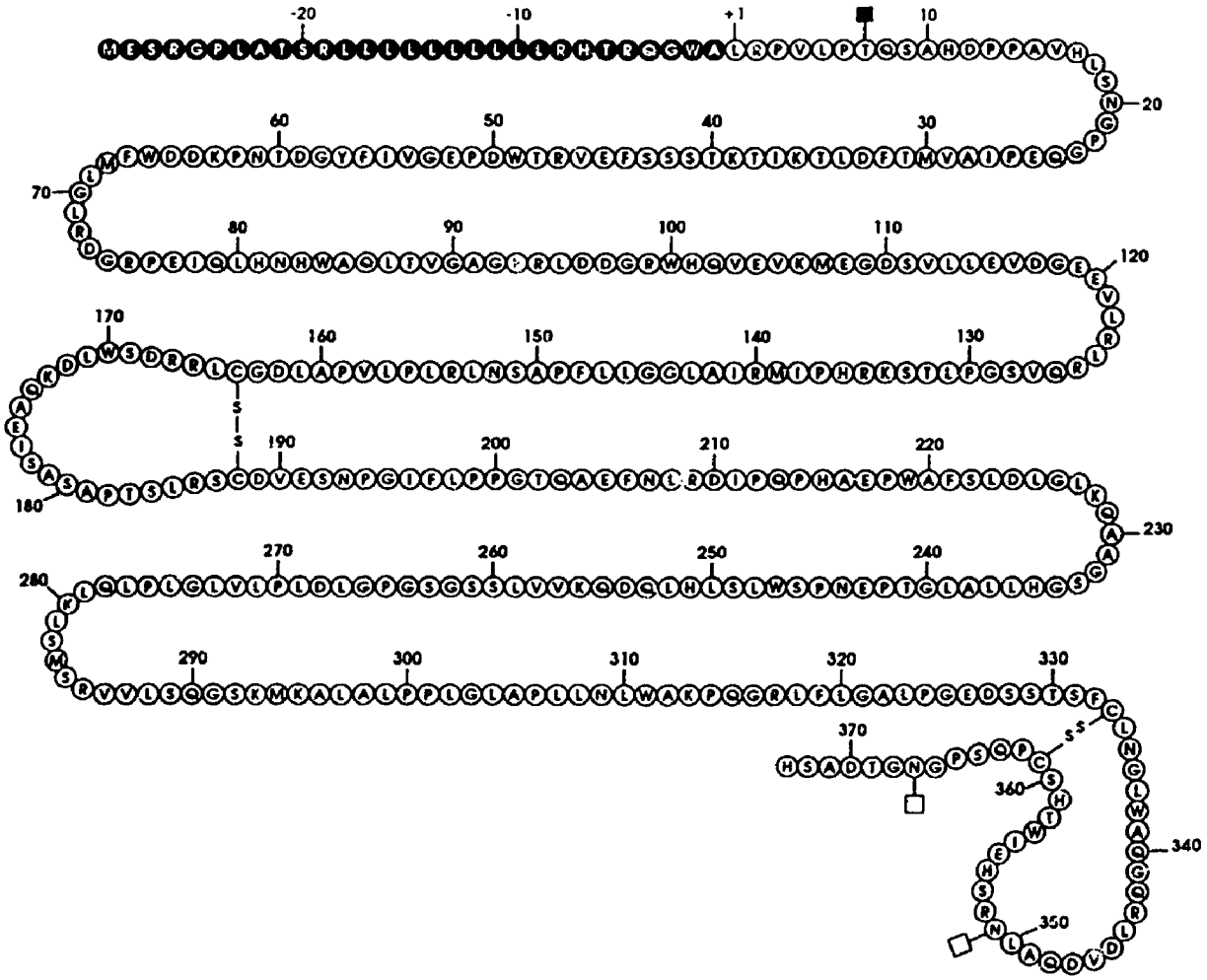
and genomic clones were isolated, which not only confirmed the primary structure of the 373 residue mature protein, but revealed that the first 29 amino acids encoded by *Shbg* comprised a hydrophobic leader sequence (Hammond *et al.*, 1987; Hammond *et al.*, 1989) (Figure 1.2).

1.4.3 Alternative Transcripts

Analysis of a human testis cDNA library revealed two other SHBG-related gene products (SHBGr-2, SHBGr,3) that diverge in nucleotide sequence at the same 5' region of the SHBG cDNA (Figure 1.1) (Hammond *et al.*, 1989). The SHBGr-2 cDNA contains a unique 5' region of 96 base pairs (bp) that is in frame with the rest of the SHBG cDNA but did not contain an initiation codon. The SHBGr-2 cDNA also contained a deletion of 208 bp corresponding to the omission of exon 7, which prematurely terminates the reading frame and replaces the final 118 amino acids of SHBG with 9 different residues. The SHBGr-3 cDNA is composed of a distinct 57 bp 5' region that contains a stop codon in frame with the remaining SHBG coding sequence. If SHBGr-3 cDNA represents an mRNA that is translated then the initiator AUG would be delineated by Met^{30}. Sequencing of human *Shbg* revealed that these two unique 5' regions are separated by only 55 bp and are approximately 1,500 bp upstream of the exon containing the initiation codon for the normal SHBG precursor (Figure 1.1), and these regions therefore represent alternative first exons (Hammond *et al.*, 1989).

Interestingly, human *Shbg* contains repetitive *Alu* sequences in the introns preceding differentially utilized exons, i.e., exons 1 and 7 (Figure 1.1) (Hammond *et al.*, 1989). These sequences have been implicated as mediators of chromosomal recombination events leading to gene rearrangements, and may also influence alternative exon usage (Lehrman *et al.*, 1987). Similarly, rat *Shbg* contains *Alu*-like sequences called repetitive DNA brain identifier (ID) elements (McKinnon *et al.*, 1987) within introns preceding alternatively used exons 1 and 6 (Joseph *et al.*, 1988; Sullivan *et al.*, 1991; Wang *et al.*, 1990). Furthermore, a cDNA was identified in a fetal rat liver library that represented a fused transcript of ABP (exons 1-5) and

Figure 1.2 The primary structure of the human SHBG precursor polypeptide. Single-letter amino acid codes are used and residues are numbered at every tenth position. The amino acids comprising the signal peptide are shown as *closed circles*. The two intramolecular disulphide linkages between paired cysteines are shown. The attachment of *O*-linked (*closed*) and *N*-linked (*open*) oligosaccharides are indicated by *squares*. The diagram is derived from Hammond, 1990.


-20
-10
+1
10
20
30
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histidine decarboxylase coding sequences (Joseph *et al.*, 1990), and which encodes a 98,000 molecular weight precursor protein. Again, two rat ID elements are present in the intervening sequence between exons 5 and 6 of the rat *Shbg* gene, adjacent to the region of this trans-splicing event (Sullivan *et al.*, 1991).

Transcripts of various sizes have been identified in RNA extracts from human tumour cells that hybridize with SHBG cDNA probes, but little is known of their structure (Mercier-Bodard *et al.*, 1991; Plymate *et al.*, 1991). It is possible that rearrangement of the gene in transformed cells results in abnormal transcripts since *Shbg* lies within an unstable region of the human genome (Hori *et al.*, 1988). However, the possible proteins encoded by these differentially spliced transcripts have never been isolated from the blood, seminal plasma or tissue homogenates, and their significance remains obscure.

1.4.4 *Shbg* Promoter

The hormonal regulation and tissue specificity of human *Shbg* expression has not yet been defined at the molecular genetic level. Typical 'TATA' or 'CAAT' box sequence elements that are commonly found in eukaryotic gene promoters are not present immediately upstream of any of the most 5' exons identified, but a liver-specific enhancer element has been identified 100 bp upstream of the translation initiation codon (AUG) for the SHBG precursor polypeptide (Hammond *et al.*, 1989). Nevertheless, the possibility remains that the promoter for the different products of *Shbg* resides further upstream (> 500 bp) of the most 5' exons identified to date.

It has been proposed that the promoter responsible for the production of rat ABP mRNA is located immediately 5' to the exon containing the initiator AUG for the precursor polypeptide. Primer extension analysis of rat ABP mRNA indicated that the transcriptional start site was 35 bp upstream of this initiation codon, and some potential regulatory elements were identified -20 bp to -340 bp from this proposed transcriptional start site (Joseph *et al.*, 1988). However, an SHBG cDNA

has recently been isolated from a sheep liver library that extends 63 bp upstream of this site, and this region exhibits 75% sequence identity to the proposed rat *Shbg* proximal promoter (Hammond, 1993). Therefore, it is still questionable whether this region may actually function as a promoter, at least in the liver.

A 5.5 kb rat genomic DNA fragment containing the entire coding region for ABP, as well as 1.5 kb region of genomic DNA upstream of the controversial transcriptional start site, was used to develop transgenic mice to aid in the identification of the promoter and enhancer regions of rat *Shbg* (Reventos *et al.*, 1993). The data demonstrated that this genomic fragment contained elements capable of directing *Shbg* expression in the testis but not the liver or brain (Reventos *et al.*, 1993). Therefore, the promoter utilized for testis-specific expression of rat ABP must be within 1.5 kb upstream from the commonly utilized first exon containing the initiation codon for Met^1. However, an alternate promoter has been identified 15 kb upstream from this 5.5 kb genomic fragment (Sullivan *et al.*, 1993). Interestingly, brain poly(A+) RNA contained rat ABP transcripts derived from only this promoter, while fetal rat liver poly(A+) RNA contained transcripts derived from both promoters. The majority of the rat ABP transcripts in the testis appears to be derived from the testis-specific promoter (Sullivan *et al.*, 1993). Therefore, it is likely that these two promoter regions may be differentially regulated in a tissue-specific fashion.

1.5 Sites of *Shbg* Expression

1.5.1 Liver

The liver (Khan *et al.*, 1981) and the testis (Hagenas *et al.*, 1975) are the major sites of SHBG synthesis in humans. Thyroid hormones and estrogens increase plasma SHBG levels *in vivo* (Anderson, 1974), while androgens (Anderson, 1974), prolactin (Lobo and Kletzky, 1983), progestins (Fotherby, 1988), insulin and insulin-like growth factor-1 (IGF-1) can decrease plasma SHBG levels (Pugeat *et al.*, 1991). *In vitro* studies using a human hepatocarcinoma cell line, HepG2, have shown that both insulin and IGF-1 decrease SHBG mRNA levels and suppress SHBG secretion

(Crave *et al.*, 1994), and that prolactin is also capable of decreasing SHBG secretion from these cells (Plymate *et al.*, 1988). Thyroid hormones increase SHBG mRNA levels (Mercier-Bodard *et al.*, 1991) and stimulate SHBG secretion from HepG2 cells (Raggatt *et al.*, 1992).

There is little doubt as to the effect of estrogens and androgens on the levels of plasma SHBG, but there is conflicting information about the influence of sex steroids on *Shbg* expression in HepG2 cells. Early studies using these cells showed that estrogens and androgens could increase SHBG secretion if the cells were grown in fetal calf serum (Lee *et al.*, 1987). However, a later study demonstrated that E_2 and tamoxifen increased SHBG mRNA, while DHT had the opposite effect (Mercier-Bodard *et al.*, 1991). It should be noted that hepatocarcinoma cell lines may be useful for these types of experiments, but they may not actually reflect the activity of *Shbg* in normal hepatocytes subjected to different hormonal backgrounds.

1.5.2 Testis

Rat *Shbg* expression has been studied in both the testis of intact animals (Reventos *et al.*, 1988) and primary Sertoli cell cultures (Joseph *et al.*, 1988). Both studies demonstrated that follicle-stimulating hormone (FSH) acts directly on Sertoli cells to increase the level of ABP mRNA, while testosterone induced ABP mRNA synthesis in intact animals only. Testosterone has been proposed to act indirectly on Sertoli cells by inducing the production of PMod-S, a peritubular cell protein, that modulates Sertoli cell function (Skinner and Fritz, 1985). Testicular ABP is secreted into the seminiferous tubules (Feldman *et al.*, 1981) and has been shown to be internalized by the epithelial cells of the caput epididymis in rats (French and Ritzen, 1973) and monkey (Gerard *et al.*, 1990) by a process that appears to involve receptor-mediated endocytosis (Pelliniemi *et al.*, 1986). In this context, ABP is thought to maintain a high androgenic milieu for the developing spermatozoa and to possibly influence the production of androgen-dependent epididymal proteins necessary for sperm maturation (Tindall and Means, 1980). It also appears that ABP interacts directly with developing sperm membranes and can be internalized,

which may alter sperm exposure to androgens in a more active fashion than previously thought (Gerard *et al.*, 1991; Gerard *et al.*, 1994). Therefore it is not surprising that homozygous transgenic male and female mice over-expressing a rat *Shbg* transgene have severely impaired fertility (Reventos *et al.*, 1993).

1.5.3 Brain

Immunoreactive ABP and its mRNA have been identified in the adult rat brain (Wang *et al.*, 1990). Several ABP-related cDNAs from a rat brain library indicate that distinctive first exons can be utilized other than the exon containing the translation initiation codon for the ABP precursor (Wang *et al.*, 1990), as in the case of the corresponding cDNAs from human testis (Hammond *et al.*, 1989) and fetal rat liver (Sullivan *et al.*, 1991) libraries. Sex steroids certainly influence sexual differentiation of the mammalian brain (Wilson, 1978; Mooradian *et al.*, 1987) and extracellular sex steroid-binding proteins may modulate this activity. Transgenic mice over-expressing rat ABP suffer gait disabilities due to a neurological defect in the central nervous system (Reventos *et al.*, 1993). This is particularly interesting because mouse *Shbg* has been mapped to chromosome 11 in a locus that contains numerous genes responsible for mouse mutants with neurological disorders (Joseph *et al.*, 1991a), one of which is also associated with male sterility (Reventos *et al.*, 1993).

1.5.4 Fetus

Fetal production of SHBG is also likely important for the growth and differentiation of tissues affected by the action of sex steroids during critical periods of development (Wilson, 1978). Although there has been one report of two sisters in whom SHBG could not be detected, the steroid-binding capacity assay used to measure serum SHBG in these patients was unable to detect SHBG at concentrations below 10 nM (Ahrentsen *et al.*, 1982). An IRMA for SHBG has subsequently been developed that is sensitive to SHBG levels well below 10 nM (Hammond *et al.*, 1985) and has been widely used for clinical diagnostic purposes for over a decade. During this period, the analysis of very large numbers of human

serum samples have failed to demonstrate a complete lack of plasma SHBG (Hammond, 1990). In addition, there has been no documented case of individuals with a plasma SHBG that exhibits compromised steroid-binding activity. The synthesis of plasma SHBG with appropriate steroid-binding properties is probably important for fetal viability. This is supported by the fact that rat *Shbg* is expressed in fetal liver at days 15-17 of gestation but the gene is quiescent in adult rat liver (Sullivan *et al.*, 1991). Furthermore, plasma ABP levels peak by 22 days in male rat pups, probably due to an incompletely formed blood-testis barrier at the onset of puberty (Cheng *et al.*, 1984) and ABP is still detectable in 60 day old male rat plasma by a radioimmunoassay (RIA) (Gunsalus *et al.*, 1978). Immunoreactive ABP has also been measured tentatively in the plasma of adult female rats by an RIA, but at a level approximately 100-fold lower than their male counterparts (Gunsalus *et al.*, 1978). Thus, these data suggest that ABP is in fact present in the plasma of adult rats, and may well be of gonadal origin.

1.5.5 Placenta

The placenta also expresses human *Shbg* as demonstrated by the presence of an SHBG mRNA in this tissue. In addition, cultured placental trophoblast cells secrete immunoreactive SHBG into the culture medium, as determined by western analysis (Larrea *et al.*, 1993). It is therefore possible that SHBG is a regulator of androgen metabolism during placental differentiation. The importance of androgen metabolism by the placenta is most dramatically illustrated by the virilization of female hyenas *in utero* (Yalcinkaya *et al.*, 1993). In these mammals, placental aromatase activity, which converts androstenedione to estrogens is limited, while 17ß-hydroxysteroid dehydrogenase, which converts androstenedione to testosterone, is very active. As a result, the hyena placenta produces very high concentrations of testosterone which virilizes the female fetus (Yalcinkaya *et al.*, 1993). Therefore, placental SHBG may be part of a protective mechanism that has evolved in some mammals to prevent female fetus masculinization by sequestering bioavailable testosterone. Potential SHBG receptors on placental cell membranes may also be part of this mechanism (see 1.10.2).

1.6 Molecular Composition of SHBG

1.6.1 Precursor Polypeptide

The nucleotide sequences of an SHBG cDNA (Hammond *et al.*, 1987) and several genomic DNA fragments (Hammond *et al.*, 1989), and the amino acid sequence of the purified plasma protein (Walsh *et al.*, 1986; Hammond *et al.*, 1986), revealed that the human SHBG precursor polypeptide is composed of 402 amino acids, with the first 29 residues comprising a hydrophobic signal peptide (Figure 1.2). Peptide sequencing indicated that the *N*-terminus of purified SHBG is heterogeneous (Hammond *et al.*, 1986; Gershagen *et al.*, 1987), and this may reflect limited proteolytic degradation or cleavage of the signal peptide in slightly different positions. Nevertheless, the mature SHBG polypeptide most likely comprises 373 amino acids with a calculated molecular weight of 40,475 and contains two disulphide bridges linking Cys^{164}-Cys^{188} and Cys^{333}-Cys^{361} (Walsh *et al.*, 1986). In addition, the locations of attachment sites for *N*-linked oligosaccharides at Asn^{351} and Asn^{367}, and an *O*-linked carbohydrate chain at Thr^{7} (Figure 1.2), have been identified (Walsh *et al.*, 1986).

1.6.2 Subunit Size Variations

Analyses of purified human SHBG from plasma revealed that it exists as a dimeric glycoprotein of 90-100 kDa (Westphal, 1986). However during polyacrylamide electrophoresis (PAGE) under denaturing conditions, plasma SHBG dissociates into a doublet of heavy (52 kDa) and light (48 kDa) subunits in an approximate 10:1 ratio. Therefore the dimer may be comprised of a mixture of subunits, with the heavy:heavy isoform predominating (Cheng *et al.*, 1985b). The detection of only one SHBG polypeptide (Hammond and Robinson, 1984; Hammond *et al.*, 1986; Petra *et al.*, 1986) together with immunochemical evidence for two identical epitopes per dimer supported the conclusion that SHBG exists as a homodimer (Hammond *et al.*, 1986). Furthermore, an electrophoretic variant of SHBG has been identified which is characterized by an additional subunit that is larger than the two subunits normally associated with SHBG (Luckock and Cavalli-Sforza, 1983; Khan *et al.*, 1985; Gershagen *et al.*, 1987). There has been

debate as to whether the subunit size heterogeneity of SHBG is due to variations in glycosylation (Danzo *et al.*, 1989) and/or modification of the polypeptide (Cheng *et al.*, 1985a), but the process of subunit association and the question of whether different combinations of isoforms are functionally important are largely unexplored.

1.6.3 Carbohydrate Composition

Methylation analysis of the carbohydrate composition of human SHBG revealed that each subunit contains two biantennary *N*-linked oligosaccharide chains of the *N*-acetyllactosamine type, and one *O*-linked oligosaccharide (Avvakumov *et al.*, 1983), which together constitute about 12% of the total mass of the glycoprotein (Hammond *et al.*, 1986). Variable amounts of sialic acid are probably responsible for the series of bands observed when SHBG is subjected to isoelectric focusing, and this is supported by the observation that the number of isoforms are greatly reduced after treatment with neuraminidase or *N*- and *O*-glycanases (Hammond *et al.*, 1986; Mischke *et al.*, 1979; Danzo *et al.*, 1989).

Concanavalin A (Con A) chromatography has been employed to resolve glycoproteins based on differences in their glycosylation. Con A is a lectin from the jack bean plant that only interacts with proteins containing at least one *N*-linked biantennary or high mannose-type carbohydrate chain (Krusius *et al.*, 1976; Narasimhan *et al.*, 1979). When human SHBG from plasma is subjected to Con A chromatography, virtually all the glycoprotein remains bound to the column, while about half the human ABP from testicular cytosols binds to this lectin (Hsu and Troen, 1978; Cheng *et al.*, 1985b). Physicochemical analyses of these proteins revealed that Con A-bound ABP is essentially identical to plasma SHBG, while the Con A-unbound form exhibits a distinct peptide map and appears to be composed of subunits that are both 2 kDa larger than those of plasma SHBG (Cheng *et al.*, 1985b). These data suggested that the protein which did not interact with Con A probably contained more branched oligosaccharide chains. It was initially thought that the Con A-binding form of ABP in testicular cytosols could reflect SHBG from contaminating blood or extravascular fluids, but similar preparations of ABP from

adult rat testes displayed a similar Con A elution profile (Cheng *et al.*, 1984). This provided evidence that two forms of human ABP were probably being synthesized by the Sertoli cells and suggests that SHBG can be differentially glycosylated, and that oligosaccharide chain attachments may be cell-type specific.

Carbohydrate chains have been implicated in the folding and solubility of glycoproteins, their intracellular trafficking and secretion, metabolic clearance and catabolism, ligand-binding ability, recognition by membrane receptors, and the induction of secondary messenger systems (Avvakumov *et al.*, 1993; Avvakumov and Hammond, 1994; Rose and Doms, 1988; Combarnous, 1992). The biological half-life of bovine SHBG in the blood circulation of rats was shown to be decreased by desialylation, perhaps due to clearance by the asialoglycoprotein receptor (Suzuki and Sinohara, 1979). Neuraminidase treatment of human SHBG did not affect its steroid-binding properties (Avvakumov *et al.*, 1988), and various electrophoretic isoforms are capable of interacting with steroids, as determined by photoaffinity labeling (Cheng *et al.*, 1985b; Danzo *et al.*, 1989). Although, enzymatic deglycosylation of SHBG has demonstrated that its steroid-binding activity remained essentially unchanged (Petra *et al.*, 1992), this type of study ignores the potential influence of carbohydrate chains on the folding of the protein during synthesis. For example, site-directed removal of glycosylation sites from human CBG has demonstrated that attachment of an oligosaccharide chain to Asn^{238} is essential for the proper folding required for the formation of its steroid-binding site during biosynthesis (Avvakumov *et al.*, 1993; Avvakumov and Hammond, 1994). Purified SHBG also binds to proteins on the plasma membranes of several sex steroid-responsive tissues and cell lines (see 1.10), and carbohydrates may play important roles in these interactions.

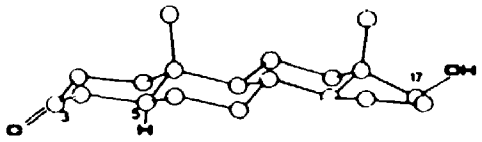
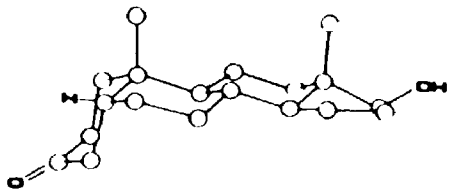
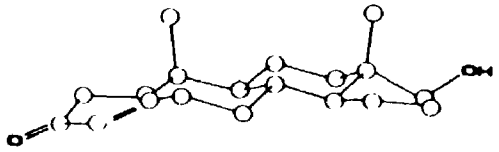
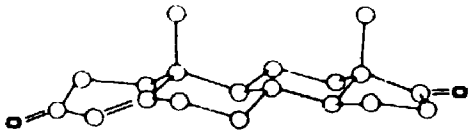
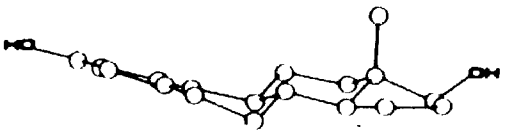
1.7 Steroid Ligand Structural Requirements

The molecular structure that best fits the SHBG steroid-binding site is DHT, as exemplified by its high affinity interaction ($k_d=10^{-10}$ M), therefore the binding properties of DHT are usually compared to other steroid ligands (Dunn *et al.*,

1981). The planarity of the steroid molecule, as indicated by an angle between ring A and the BCD ring plane (Duax and Norton, 1975) has a dramatic impact on its affinity for SHBG (Table 1.2) (Westphal, 1986). The A/B ring angle in DHT is 11° compared to 65° for 5ß-DHT, which results in a 100-fold decrease in affinity for SHBG. Furthermore, the presence of double bonds at C4 (testosterone) or C5 (androst-5-ene-3ß,17ß-diol) reduces the affinity for SHBG by altering the angle of the steroid A ring, which is 23° for testosterone (Westphal, 1986; Dunn *et al.*, 1981). A 17ß-OH group is also required for strong binding to SHBG, as demonstrated by the impaired binding of steroids that contain a 17α-OH or a 17-ketone group (Cunningham *et al.*, 1981; Dunn *et al.*, 1981). This effect is best exemplified by the loss of binding affinity for androstenedione (Dunn *et al.*, 1981) which differs from testosterone by a 17-ketone in place of a 17ß-OH (Table 1.2). A ketone at C3 is also important for optimum binding to SHBG, but an OH group is tolerated with only a 3-fold loss in affinity, as exemplified by comparing the relative binding affinities of DHT and 5α-androstane-3ß,17ß-diol (Cunningham *et al.*, 1981; Dunn *et al.*, 1981). Furthermore, some C18 steroids including 17ß-estradiol can also bind to SHBG but with markedly reduced affinity (Table 1.2). In general, optimal steroid binding to SHBG appears to require a planar C19 steroid with a 17ß-OH group, and an electronegative functional group at C3 (Cunningham *et al.*, 1981).

Some synthetic steroids can compete with endogenous ligands for the SHBG steroid-binding site (Pugeat *et al.*, 1981), and may interfere with the role of SHBG in modulating sex steroid physiology (Hammond *et al.*, 1994). Therefore, the molecular requirements of a steroid ligand for optimum binding to SHBG have been examined in great detail (Cunningham *et al.*, 1981; Westphal, 1986). This knowledge of the structural characteristics of various SHBG ligands has been important for the design of new synthetic steroids with improved efficacy such as the development of contraceptive progestins (Hammond *et al.*, 1994).

Table 1.2 Comparison of the structural properties of various steroids and the impact on the binding affinity of SHBG. The carbon atom backbone in each steroid-conformation diagram is represented by *open circles*. Diagrams were derived from Duax and Norton, 1975 and ring angle data were from Westphal, 1986.

STEROID	STRUCTURE	A/B RING ANGLE	RELATIVE BINDING AFFINITY
5α-DHT		11.0°	100.0
5ß-DHT		65.1°	0.1
Testosterone		23.4°	20.0
Androstenedione		-	0.1
17ß-Estradiol		-	5.0

1.8 SHBG Steroid-binding Site

1.8.1 Models For Steroid-binding

Although the steroid ligand structures required for high affinity binding to SHBG have been determined, little is known about the molecular composition of the steroid-binding site. Monomers of SHBG do not bind steroid (Casali *et al.*, 1990) and it is generally agreed that the two subunits must somehow associate to form a single steroid-binding site. Two models have been proposed to account for these data (Petra, 1991), but both are speculative in the absence of tertiary structure information. One model for negative cooperativity states that the steroid binds to one subunit and causes a conformational change in the other to prevent the binding of a second steroid. The other more likely model states that both subunits contribute structural elements to the steroid-binding domain, and that the ligand resides at an interface between the subunits (Petra, 1991).

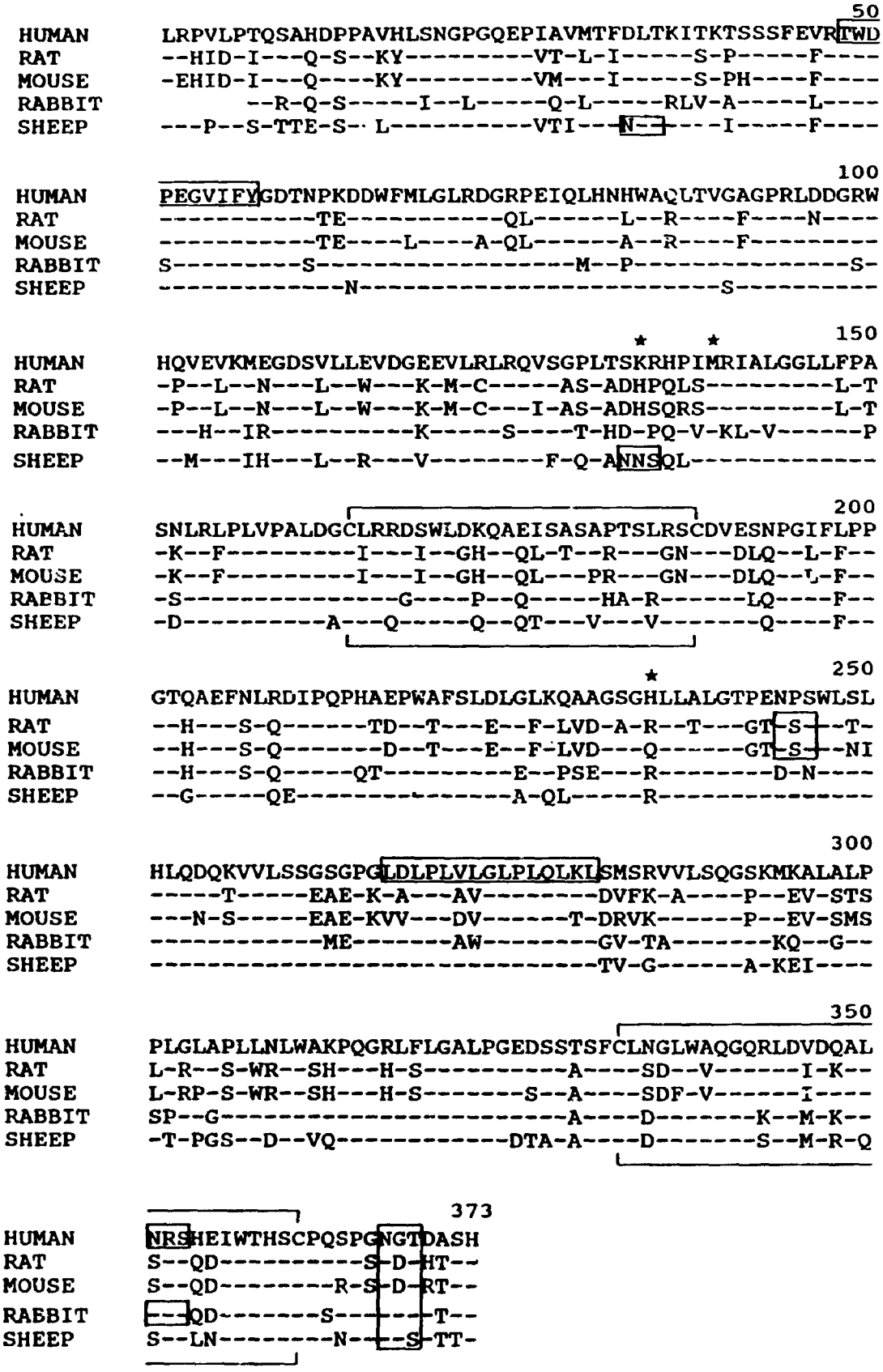
1.8.2 Photoaffinity Labeling With Δ6-[^{3}H]Testosterone

Affinity labeling techniques have been used to show that various subunit isoforms of SHBG interact with steroid (Cheng *et al.*, 1983; Schmidt *et al.*, 1981). Once the primary structure of human SHBG was resolved, it was possible to identify regions or specific amino acids that might interact with affinity ligands. In two independent studies using Δ6-[^{3}H]testosterone (Δ6-T) as photoaffinity ligand, a *C*-terminal fragment of human SHBG beginning at Ala^{296} was shown to interact with the ligand (Hammond *et al.*, 1987; Petra *et al.*, 1988). However, more convincing data using Δ6 derivatives of both T and E_2, identified Met^{139} (Figure 1.3) as the photoaffinity-labeled residue in each case (Grenot *et al.*, 1988; Grenot *et al.*, 1992). In addition, a region of rat ABP analogous to residues 141-151 in human SHBG (Figure 1.3), was also labeled with Δ6-T, providing further evidence for a more *N*-terminal location of the steroid-binding domain (Danzo *et al.*, 1991).

1.8.3 Affinity Labeling With 17ß-bromoacetoxy-DHT

Another affinity label, 17ß-bromoacetoxy-DHT, has been used to identify amino acids in human SHBG that interact with steroid ligands (Figure 1.3). Again,

Figure 1.3 Comparis of the primary structures of sex steroid-binding proteins in different mammalian species. Single letter amino acid codes are used and a *dash* indicates identity to the human SHBG sequence. *Stars* indicate amino acids in human SHBG that have been labeled by affinity ligands. The putative receptor-binding domain (residues 48-57) and the region of alternating leucines (residues 267-281) in human SHBG are *boxed*. Consensus sites for *N*-glycosylation are also indicated in this manner. The cysteines that form disulphide bridges are indicated in pairs by a *line*. The diagram is derived from Hammond, 1993.


50
HUMAN LRPVLPTQSAHDPPAVHLSNGPGQEPIAVMTFDLTKITKTSSSFEVRTWD
RAT --HID-I---Q-S--KY---------VT-L-I-----S-P-----F----
MOUSE -EHID-I---Q----KY---------VM---I-----S-PH----F----
RABBIT --R-Q-S-----I--L-----Q-L-----RLV-A-----L----
SHEEP ---P--S-TTE-S- L----------VTI---N---I-----F----
100
HUMAN PEGVIFYGDTNPKDDWFMLGLRDGRPEIQLHNHWAQLTVGAGPRLDDGRW
RAT ----------TE----------QL------L--R----F----N----
MOUSE ----------TE----L----A-QL------A--R----F---------
RABBIT S---------S----------M--P----------S-
SHEEP ----------N----------S----------
150
HUMAN HQVEVKMEGDSVLLEVDGEEVLRLRQVSGPLTSKRHPIMRIALGGLLFPA
RAT -P--L--N---L--W---K-M-C-----AS-ADHPQLS---------L-T
MOUSE -P--L--N---L--W---K-M-C---I-AS-ADHSQRS---------L-T
RABBIT ---H--IR----------K-----S----T-HD-PQ-V-KL-V------P
SHEEP --M---IH---L--R---V--------F-Q-ANNSQL----------
200
HUMAN SNLRLPLVPALDGCLRRDSWLDKQAEISASAPTSLRSCDVESNPGIFLPP
RAT -K--F---------I---I--GH--QL-T--R---GN---DLQ--L-F--
MOUSE -K--F---------I---I--GH--QL---PR---GN---DLQ--L-F--
RABBIT -S----------G----P--Q-----HA-R------LQ----F--
SHEEP -D----------A---Q-----Q--QT---V---V-------Q----F--
250
HUMAN GTQAEFNLRDIPQPHAEPWAFSLDLGLKQAAGSGHLLALGTPENPSWLSL
RAT --H---S-Q------TD--T---E--F-LVD-A-R--T---GT-S---T-
MOUSE --H---S-Q-------D--T---E--F-LVD---Q------GT-S---NI
RABBIT --H---S-Q-----QT---------E--PSE---R--------D-N----
SHEEP --G-----QE----------A-QL-----R----------
300
HUMAN HLQDQKVVLSSGSGPGLDLPLVLGLPLQLKLSMSRVVLSQGSKMKALALP
RAT -----T-----EAE-K-A---AV--------DVFK-A----P--EV-STS
MOUSE ---N-S-----EAE-KVV---DV------T-DRVK------P--EV-SMS
RABBIT ----------ME-------AW--------GV-TA-------KQ--G--
SHEEP ----------TV-G------A-KEI----
350
HUMAN PLGLAPLLNLWAKPQGRLFLGALPGEDSSTSFCLNGLWAQGQRLDVDQAL
RAT L-R--S-WR--SH---H-S----------A----SD--V------I-K--
MOUSE L-RP-S-WR--SH---H-S-------S--A----SDF-V------I----
RABBIT SP--G----------A----D-------K--M-K--
SHEEP -T-PGS--D--VQ----------DTA-A----D-------S--M-R-Q
373
HUMAN NRSHEIWTHSCPQSPGNGTDASH
RAT S--QD----------S-D-HT--
MOUSE S--QD--------R-S-D-RT--
RABBIT ---QD-------S-------T--
SHEEP S--LN--------N-----S-TT-

conflicting results were obtained since His^{235} was identified as the labeled residue by one group (Khan and Rosner, 1990), while Lys^{134} was found to be labeled by another (Namkung *et al.*, 1990). Although this affinity ligand labeled different amino acids that are distant from one another on a linear array, the topography of the folded polypeptide could orient distant residues into close proximity, and different regions of the molecule could participate in forming a steroid-binding site. However, the nature of this affinity ligand, due to the attachment of an unnatural bulky reactive group to the steroid, casts doubt as to which amino acid(s) is actually closest to the steroid ligand (Grenot *et al.*, 1992).

1.8.4 Proposed Locations for the Steroid-binding Domain

A comparison of the amino acid sequences of several steroidogenic enzymes and steroid receptors revealed a consensus sequence that is similar to a region spanning residues 107-130 in human SHBG and rat ABP (Figure 1.3), and the immediate interpretation was that it might constitute part of the steroid-binding pocket (Picado-Leonard and Miller, 1988). This consensus sequence was subsequently deleted from the estrogen receptor and did not effect its steroid-binding activity, which casts doubt on its biological significance (Fawell *et al.*, 1989). In addition, an unusual stretch of alternating leucines occurs between residues 267-281 in SHBG (Figure 1.3), and it was suggested that this region might form a novel secondary structure that could accommodate a steroid (Walsh *et al.*, 1986).

1.9 Dimerization of Subunits

Little is known about the mechanism of subunit association and whether specific region(s) of each monomer contain dimerization domains that participate in protein-protein interactions. It is known that there are two intramolecular disulphide bridges within each monomer and that the two subunits are not covalently linked. Presumably, during synthesis, monomers either begin to associate with each other in a co-translational manner, or each monomer folds independently and then dimerizes. Again, the attachment of carbohydrate chains in either situation may be required prior to completion of the final folded state, as in the case of CBG

(Avvakumov and Hammond, 1994). Furthermore, the orientation of the monomers relative to each other has not been determined.

Fluorescence spectroscopy of rabbit SHBG demonstrated that the extent of subunit dissociation caused by guanidinium chloride treatment could be reduced if DHT was present in solution. Furthermore, denatured SHBG could be partially renatured in the presence of DHT, suggesting that steroid ligands contribute to the energy of dimerization (Casali *et al.*, 1990). Another study demonstrated that human SHBG partially purified from serum developed a rapid and irreversible loss of steroid-binding capacity upon storage. In an attempt to account for this, it was found that Ca^{2+} and DHT could partially prevent the loss of SHBG binding capacity due to thermal inactivation (Rosner *et al.*, 1974). The conclusion drawn from these results is that these agents help maintain the integrity of the steroid-binding domain directly, or indirectly by enhancing dimer stability. In addition, evidence for four metal-binding sites per rabbit and human SHBG dimer were found using the luminescent probe, terbium, however the location and affinity of these sites have not been determined (Ross *et al.*, 1985), and the role of metals in SHBG dimerization or steroid binding remains obscure.

It has been assumed that steroid binding and dimerization are linked processes because it has never been shown conclusively that an SHBG monomer can bind steroid. The renaturation experiments mentioned above suggest that the steroid could provide a conformational template for two monomers to dimerize during synthesis (Casali *et al.*, 1990). However, a study on the interaction of [^{125}I]SHBG with unlabeled SHBG immobilized on agarose detected specific binding between labeled and unlabeled protein with a $k_d = 10^{-12}$ M (Strel'chyonok and Avvakumov, 1990). This result suggested that the affinity of one subunit for another is greater than the affinity of the dimer for DHT by at least two orders of magnitude, and that dimers would form in the absence of steroid. This apparent uncertainty has not been resolved because it has not been possible to disrupt steroid binding or dimerization independently. Finally, a dimerization domain has not been identified

in SHBG, but the region containing a series of alternating leucines between residues 267-281 (Figure 1.3) has been postulated to also serve this function (Petra, 1991).

1.10 Plasma Membrane Protein Interactions

1.10.1 Intracellular Localization of SHBG

The immunochemical localization of sex steroid-binding proteins in human (Egloff *et al.*, 1982), monkey (Bordin and Petra, 1980) and rat (Pelliniemi *et al.*, 1981) male reproductive tissues provided the first evidence that these proteins could interact directly with sex steroid-responsive cells. As expected, ABP is present in the Sertoli cells of the testis and is bound to developing spermatocytes, but more importantly ABP is found within the epithelial cells of the proximal caput epididymis (French and Ritzen, 1973; Pelliniemi *et al.*, 1986; Egloff *et al.*, 1982). In addition, ABP has been localized to the cells of the monkey and rat prostate (Bordin and Petra, 1980; Larriva-Sahd *et al.*, 1991), and these observations suggested that ABP could be selectively internalized by sex steroid-dependent cells.

The sex hormone-dependent nature of breast cancer provided the impetus for immunocytochemical studies of human mammary carcinoma tissues and cell types, which revealed the intracellular localization of SHBG (Tardivel-Lacombe *et al.*, 1984; Bordin and Petra, 1980). Only MCF-7 human mammary carcinoma cells that were cultured in the presence of exogenous SHBG displayed a cytoplasmic location for SHBG, although *de novo* synthesis of SHBG could not be entirely ruled out (Bordin and Petra, 1980). Later studies demonstrated that MCF-7 cells do not contain SHBG mRNA (Mercier-Bodard *et al.*, 1991), supporting the claim that SHBG could be internalized by breast cells *in vivo* and *in vitro* (Tardivel-Lacombe *et al.*, 1984; Bordin and Petra, 1980). Subsequent studies confirmed that SHBG can be internalized by MCF-7 cells *via* receptor-mediated endocytosis (Porto *et al.*, 1991) and that SHBG specifically interacts with protein(s) on the plasma membranes of these cells (Porto *et al.*, 1992b).

1.10.2 Plasma Membrane Receptors For SHBG

Once it became evident that SHBG could enter the cytoplasm of cells that did not express it, other sex steroid-responsive tissues were examined for the presence of a plasma membrane receptor for SHBG. Binding sites for SHBG were first demonstrated on plasma membrane preparations from human decidual endometrium (Strel'chyonok *et al.*, 1984b). The endometrial membrane was found to exhibit a high affinity ($k_d=10^{-12}$ M) for SHBG complexed with E_2, while unliganded SHBG or SHBG bound with T or DHT was not recognized by this system (Avvakumov *et al.*, 1986; Strel'chyonok and Avvakumov, 1990). The endometrium is an estrogen target tissue and these data suggest that endometrial cells only interact with SHBG molecules exhibiting a particular conformation induced by the binding of estrogen. Furthermore, it was shown that the removal of *N*-acetylneuraminic acid from SHBG resulted in a complete loss of its ability to bind specifically to these membranes (Avvakumov *et al.*, 1988). These results demonstrated that carbohydrate chains are important determinants for recognition by membrane-binding sites either by interacting directly with the 'receptor', or by causing a conformational change in the polypeptide to affect this recognition. Later, the same group demonstrated that another specific recognition system existed on placental syncytiotrophoblast membranes which only bound SHBG complexed with T or DHT with a $k_d=10^{-12}$ M (Krupenko *et al.*, 1990). Again, these results seemed physiologically relevant since the placenta is a steroidogenic tissue capable of converting androgens to estrogens.

Human prostate tissue has also been studied extensively for plasma membrane SHBG binding sites (Hryb *et al.*, 1985) and initial experiments demonstrated that SHBG could bind specifically to these membranes with modest affinity ($k_d=1.5 \times 10^{-7}$ M). Solubilization of this receptor increased its affinity for SHBG 10-fold and also permitted the estimation of its molecular weight at 170,000 (Hryb *et al.*, 1989) and a similar size was also determined for a solubilized testicular SHBG receptor (Porto *et al.*, 1992a). Further characterization of SHBG/receptor interactions in the human prostate revealed that only unliganded SHBG could bind to this receptor ($k_d=8.6$

x 10^{-10} M), and that this process could be inhibited by the presence of steroid ligands for SHBG with a potency that closely reflects their affinity for SHBG (Hryb *et al.*, 1990). Moreover, receptor-bound SHBG appears to interact with DHT with no substantive difference in affinity when compared to non-receptor bound SHBG. In summary, the authors proposed that only unoccupied SHBG could bind to the receptor and then receptor-bound SHBG could interact with a steroid ligand, which might activate a second messenger system, induce internalization of the steroid/SHBG/receptor complex, or cause dissociation of SHBG from the receptor (Hryb *et al.*, 1990).

1.10.3 Potential Receptor-binding Domain

A potential receptor-binding domain within the SHBG molecule was defined by the ability of SHBG-derived tryptic peptides to compete with [^{125}I]SHBG for binding to the prostate plasma membrane receptor. Only a single peptide corresponding to residues 48-57 of SHBG was found to inhibit receptor binding, albeit with four orders of magnitude lower affinity (Khan *et al.*, 1990), and the fact that this span of residues is almost perfectly (98%) conserved between human, rat, mouse, sheep and rabbit sex steroid-binding proteins (Figure 1.3) suggests that it indeed represents an important region of the molecule.

1.10.4 Second Messenger System

Initial studies of the prostate-derived SHBG receptor using LNCaP cells, a human prostatic carcinoma epithelial cell line, demonstrated that unliganded SHBG binds to the plasma membrane and induces cAMP levels 2-fold upon the addition of the biologically active steroids, DHT and E_2 (Nakhla *et al.*, 1990). Conversely, cells initially treated with unliganded SHBG and then incubated with 2-methoxyestradiol, a biologically inert steroid that binds to SHBG with greater affinity than T or E_2, did not affect cAMP levels (Nakhla *et al.*, 1990). However, recent work with benign prostatic hypertrophy tissue revealed that E_2, but not DHT, acted in concert with SHBG to produce an 8-fold increase in intracellular cAMP (Nakhla *et al.*, 1994). Surprisingly, the addition of DHT prior to E_2 completely

negated this effect, and cAMP induction was not blocked by tamoxifen, an estrogen antagonist. Furthermore, this response was not seen with diethylstilbestrol, an estrogen agonist, which excluded the classical estrogen receptor pathway as a mediator of this effect (Nakhla *et al.*, 1994). This activity was localized to the prostate stromal cells, which may account for the different results obtained using LNCaP epithelial cells (Nakhla *et al.*, 1990).

In summary, specific, high affinity plasma membrane-binding sites for SHBG have been characterized in several tissues. The affinity of these interactions and the generation of second messengers appears to vary depending on the cell type or tissue studied, as well as the state of occupancy of the SHBG steroid-binding site. Although, the identification of the SHBG receptor and the downstream biochemical events that are initiated by membrane binding remain obscure, it is apparent that sex steroid-binding proteins and their receptors may play a more active role in determining the activity of steroids in specific cell types.

1.11 Homologous Proteins

1.11.1 Mammalian Sex Steroid-binding Proteins

The primary structures of the sex steroid-binding proteins from five species (Hammond *et al.*, 1987; Walsh *et al.*, 1986; Sui *et al.*, 1992; Wang *et al.*, 1989; Griffin *et al.*, 1989; Hammond, 1993) have been determined (Figure 1.3), and the percent amino acid sequence identity relative to human SHBG are indicated; rat (68%), mouse (67%), rabbit (79%) and sheep (80%). Generally, these proteins bind steroid ligands with the same relative affinity, DHT > T > E_2, but differ between species with respect to the affinity for each steroid (Westphal, 1986). Rat and mouse ABP bind androgens and estrogens with 5-10 fold lower affinity than human SHBG (Tindall and Means, 1980), while rabbit and sheep SHBG exhibit affinities for sex steroids that are 3-fold lower than human SHBG (Westphal, 1986). Furthermore, these extracellular sex steroid-binding proteins exhibit no obvious similarities in primary structure with other plasma steroid-binding proteins, the nuclear sex steroid receptors (Bardin *et al.*, 1988), or the steroidogenic enzymes

(Picado-Leonard and Miller, 1988).

1.11.2 Conserved Structural Features

Structural features in these plasma sex steroid-binding proteins that have been conserved throughout evolution include the four cysteines that compose the two intramolecular disulphide bridges (Figure 1.3). The consensus site for *N*-linked oligosaccharide attachment at Asn^{367} is also invariably present (Khan *et al.*, 1990; Avvakumov *et al.*, 1988). Only human SHBG has been shown to contain an *O*-linked carbohydrate chain at Thr^{7} and its biological significance is unknown. Of the amino acids labeled by affinity ligands, only Met^{139} is present in all species (Figure 1.3), and this suggests that it is important and participates in steroid-binding.

1.11.3 Protein S

Protein S is a plasma protein that binds to cell surfaces and interacts with Protein C as a cofactor in the regulation of the blood coagulation system (Esmon, 1987). However, unlike many of the other clotting factors, the *C*-terminal portion of Protein S is not a serine protease (Furie and Furie, 1988), and surprisingly displays some weak sequence identity (26%) with human SHBG (Baker *et al.*, 1987; Joseph and Baker, 1992). Furthermore, the SHBG receptor-binding domain exhibits 70% homology with the corresponding ten residues in Protein S (Figure 1.4). More intriguing is the association between sex steroids and disorders of blood coagulation, such as thrombosis and atherosclerosis (Poller, 1978), and the fact that individuals with Protein S deficiency are predisposed towards venous thrombosis (Schwarz *et al.*, 1984; Engesser *et al.*, 1987). Another interesting point to note is that synthetic inhibitors and substrates of serine proteases can inhibit the binding of steroids to SHBG, and it has been proposed that the tertiary structure of steroid-binding proteins and proteases are somehow related (Baker *et al.*, 1987).

1.11.4 Laminin A and Merosin

Regions of SHBG, and in particular residues within and encompassing the putative receptor-binding domain, share sequence homology with two extracellular

Figure 1.4 Comparison of the primary sequence of the putative SHBG receptor-binding domain with homologous regions of other human proteins. Single-letter amino acid codes are used and a *dash* indicates identity to the SHBG sequence. The first r sidue in each sequence is *numbered*.

PROTEIN	N-TERMINAL RESIDUE #	PRIMARY SEQUENCE
SHBG	44	F E V R T W D P E G V I F Y
PROTEIN S	286	- D F - - Y - S - - - - - L -
LAMININ A	346	L S I - - L A S S - L - Y -
MEROSIN	811	L - - - - E A E S - L - - -

matrix proteins, namely the laminin A chain and merosin (Figure 1.4) (Joseph and Baker, 1992). Again, this observation is important because laminin A and merosin both interact with plasma membrane-binding proteins (Gehlsen *et al.*, 1992; Engvall *et al.*, 1992), including several members of the integrin family of cell surface receptors (Hynes, 1992). Furthermore, the SHBG receptor isolated from prostate (Hryb *et al.*, 1989) and testicular (Porto *et al.*, 1992a) cell membranes appears to have a molecular size of approximately 170,000, which is very similar to the size of several subunits that comprise the integrin receptors (Hynes, 1987; Albelda and Buck, 1990). These observations support suggestions that SHBG may not only modulate sex steroid action by regulating their plasma distribution, but could influence blood coagulation, as well as the growth and differentiation of tissues in developing mammals by interacting directly with cell membranes.

1.12 Objectives of Study

As a first step in determining the biological role of SHBG, the structural and functional properties of this protein were analyzed and a molecular biological approach was used to:

1) Design expression systems to produce wild-type and mutant forms of human SHBG in both mammalian and prokaryotic host cells and to analyze the properties of the recombinant products.
2) Utilize site-directed mutagenesis to disrupt the sites of glycosylation in human SHBG in order to selectively prevent carbohydrate attachment and to study the impact of this on its steroid binding, dimerization and secretion from mammalian cells.
3) Identify specific regions and/or amino acids of human SHBG required for its high affinity steroid-binding and dimerization properties.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

Molecular biology grade chemicals were purchased mainly from BDH, Sigma and Fisher. DNA restriction and modifying enzymes were obtained from GIBCO BRL or Promega. Radiolabeled compounds were purchased from either Dupont NEN ([1,2-^{3}H]5α-dihydrotestosterone, [^{125}I]Bolton-Hunter reagent) or Amersham ([α-^{35}S]dATP, [α-^{32}P]dCTP). Radioinert steroids were purchased from Steraloids or supplied by Dr. W. Khalil. Tissue culture media, fetal bovine serum (FBS), antibiotics, Geneticin (G418), and trypsin-EDTA were purchased from GIBCO BRL. Oligonucleotide primers were synthesized by the Molecular Biology Core Facility of the Medical Research Council of Canada Group in Fetal and Neonatal Health and Development. The SHBG immunoradiometric assay (IRMA) kits were donated by Orion Diagnostica. Out-dated pregnancy serum (Blood Bank, Victoria Hospital Corporation, London, Ontario) was utilized as the source of human SHBG unless otherwise stated.

2.1.2 Plasmids and Bacterial Strains

Cloning vectors employed in this study are pBR322, pBluescript (Stratagene) and pT7/T3-18 (GIBCO BRL). The pSELECT vector (Altered Sites, Promega) was used to introduce nucleotide changes in the human SHBG cDNA for the mutagenesis studies. The pGEX-2T vector (Pharmacia) was utilized for expressing glutathione S-transferase (GST) fusion proteins in bacteria. Vectors used for the expression of proteins in mammalian cells were pRc/CMV (Invitrogen) and pBPV (Pharmacia). The pSV2neo vector (Clontech) was co-transfected with pBPV. The *Escherichia coli* strain BMH71-18 mutS was used for mutagenesis (Altered Sites, Promega), and the JM107 strain for expression of GST fusion proteins or routine plasmid amplification.

2.1.3 Mammalian Cell Culture

Chinese hamster ovary (CHO pro^{-}, wild type) cells (Chaney *et al.*, 1986) and BW-1 mouse hepatoma cells (Szpirer and Szpirer, 1975) were donated by Dr. W.

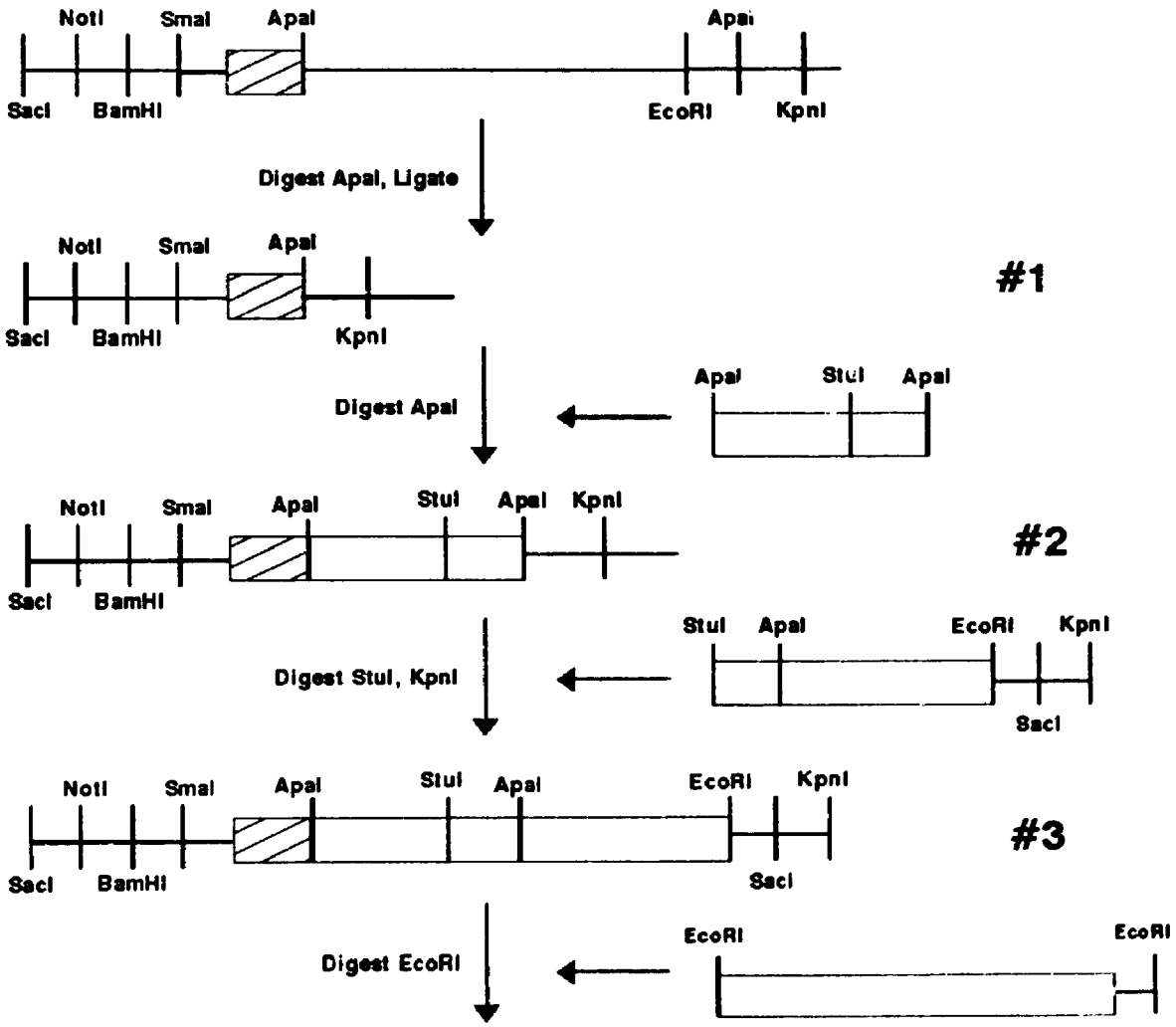
Flintoff and Dr. J. Koropatnick, respectively. Both cell lines were maintained at 37°C in 5% CO_2 and routinely cultured in α-minimal essential medium containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), penicillin (5 units/mL), streptomycin (5 mg/mL) and 10% FBS (MEM/FBS) and harvested with 0.25% trypsin/1 mM EDTA.

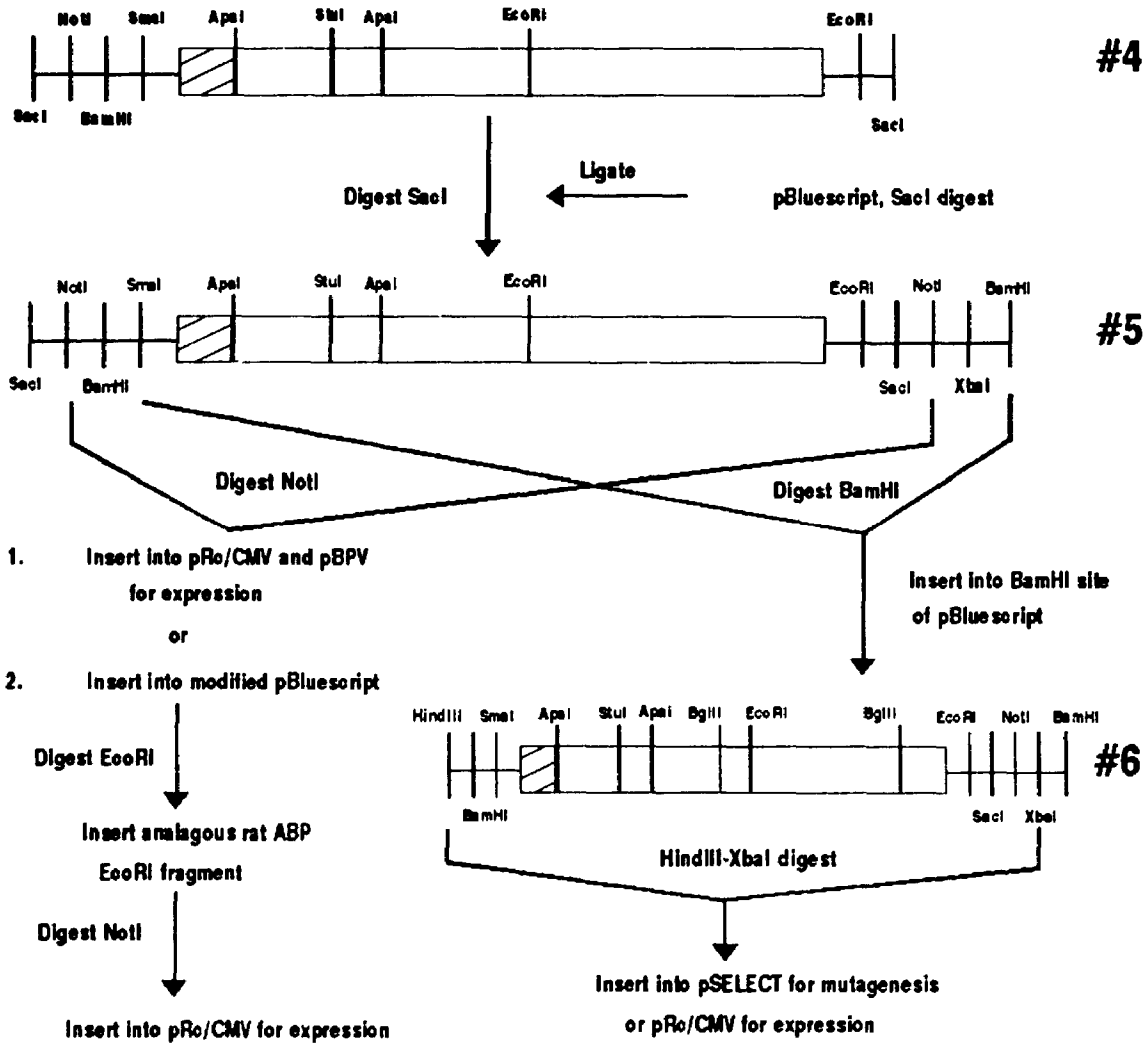
2.2 Generation of cDNA Expression Constructs

2.2.1 Human SHBG cDNA *Not*I Fragment

A 1,105 base pair (bp) *Sma*I-*Eco*RI fragment of human genomic DNA that contains the exon encoding the complete secretion signal polypeptide sequence (Hammond *et al.*, 1989) was inserted into pBluescript. A 189 bp *Sma*I-*Apa*I fragment containing the initiation codon and 98 bp of 5' flanking sequence were selectively maintained in pBluescript by digestion with *Apa*I and re-ligation of the vector (Figure 2.1, construct #1). An SHBG 5' cDNA clone in pT7/T3-18 was digested with *Apa*I to remove a 254 bp *Apa*I SHBG cDNA fragment (Hammond *et al.*, 1987), containing an internal *Stu*I site. This 254 bp fragment was purified by low melting agarose-gel electrophoresis (Wieslander, 1979), and inserted into the *Apa*I site of construct #1 in the correct orientation, to create construct #2 (Figure 2.1). The SHBG 5' cDNA clone in pT7/T3-18 was digested with *Stu*I and *Kpn*I, removing a 391 bp *Stu*I-*Kpn*I cDNA fragment containing an internal *Eco*RI site (Hammond *et al.*, 1987), that was directionally cloned into construct #2 to create construct #3 (Figure 2.1). The remaining 555 bp of the SHBG open reading frame, contained within an SHBG cDNA *Eco*RI fragment from the original pBR322 cloning vector (Hammond *et al.*, 1987), was inserted into the *Eco*RI-digested construct #3 in the correct orientation to yield a 1,346 bp *Sma*I-*Eco*RI human SHBG cDNA construct in pBluescript (Figure 2.1, construct #4). The complete human SHBG cDNA in construct #4 is flanked by *Sac*I sites, and it was removed by digestion with *Sac*I and inserted into the *Sac*I site of an unmodified pBluescript to generate *Not*I sites flanking the SHBG cDNA (Figure 2.1, construct #5). Construct #5 was digested with *Not*I to isolate the open reading frame for the SHBG precursor polypeptide in a single cDNA fragment. This 1403 bp *Not*I

Figure 2.1 Flow diagram describing the construction of a complete human SHBG cDNA and hybrid cDNAs used for mutagenesis and/or expression in mammalian cells. The open reading frame for human SHBG is *boxed* and the region encoding the signal peptide is shown as a *hatched* box. The partial or complete cDNA constructs referred to in the text are *numbered* and the restriction endonuclease sites utilized in their construction are indicated.


NotI
SmaI
ApaI
ApaI
SacI
BamHI
EcoRI
KpnI
Digest ApaI, Ligate
NotI
SmaI
ApaI
SacI
BamHI
KpnI
#1
ApaI
StuI
ApaI
Digest ApaI
NotI
SmaI
ApaI
StuI
ApaI
KpnI
SacI
BamHI
#2
StuI
ApaI
EcoRI
KpnI
SacI
Digest StuI, KpnI
NotI
SmaI
ApaI
StuI
ApaI
EcoRI
KpnI
SacI
BamHI
SacI
#3
EcoRI
EcoRI
Digest EcoRI


NotI
SmaI
ApaI
StuI
ApaI
EcoRI
EcoRI
SacI
BamHI
SacI
#4
Digest SacI
Ligate
pBluescript, SacI digest
NotI
SmaI
ApaI
StuI
ApaI
EcoRI
EcoRI
NotI
BamHI
SacI
BamHI
SacI
XbaI
#5
Digest NotI
Digest BamHI
1. Insert into pRc/CMV and pBPV for expression
or
2. Insert into modified pBluescript
Digest EcoRI
Insert analagous rat ABP EcoRI fragment
Digest NotI
Insert into pRc/CMV for expression
Insert into BamHI site of pBluescript
HindIII
SmaI
ApaI
StuI
ApaI
BglII
EcoRI
BglII
EcoRI
NotI
BamHI
BamHI
SacI
XbaI
#6
HindIII-XbaI digest
Insert into pSELECT for mutagenesis or pRc/CMV for expression

fragment was sub-cloned into the *Not*I sites of the pRc/CMV and pBPV vectors in the correct orientation for expression of human SHBG in mammalian cells.

2.2.2 Human SHBG cDNA *Hin*dIII-*Xba*I Fragment

A second human SHBG cDNA construct was created with a 5' *Hin*dIII site and 3' *Xba*I site flanking the cDNA to facilitate its cloning into pRc/CMV for expression, and pSELECT for mutagenesis (see 2.2.5). To accomplish this, the intact SHBG cDNA in pBluescript flanked by *Not*I sites (Figure 2.1, construct #5) was used because it is also conveniently flanked by *Bam*HI sites. Therefore, after digestion with *Bam*HI, the complete SHBG cDNA fragment was inserted into the *Bam*HI site of another unmodified pBluescript vector in an orientation such that the cDNA was then flanked by *Hin*dIII and *Xba*I sites at its 5' and 3' ends, respectively (Figure 2.1, construct #6). After excision with *Hin*dIII and *Xba*I, the SHBG cDNA was then inserted into pRc/CMV for expression or pSELECT for mutagenesis.

2.2.3 Human SHBG/Rat ABP Hybrid cDNA-1

A *Not*I fragment containing the human SHBG precursor cDNA (Figure 2.1, construct #5) was sub-cloned into the *Not*I site of a pBluescript vector that was modified as follows: The *Eco*RI recognition sequence in the multiple cloning site was removed by digestion with *Eco*RV and *Sma*I and by blunt-end ligating the vector ends. The SHBG cDNA construct was digested with *Eco*RI to remove a 555 bp fragment from its 3' end and this was replaced with the corresponding 3' rat ABP cDNA fragment (Reventos *et al.*, 1988). This hybrid cDNA was excised and inserted into the *Not*I site of pRc/CMV for expression of a mature chimeric protein which is composed of 205 *N*-terminal amino acids encoded by the human cDNA and the remaining 168 residues encoded by the rat cDNA (Figure 5.1).

2.2.4 Human SHBG/Rat ABP Hybrid cDNA-2

A second human SHBG/rat ABP hybrid cDNA was constructed by digesting the human SHBG cDNA in pBluescript (Figure 2.1, construct #6) with *Stu*I and *Bgl*II

to remove a 306 bp *Stu*I-*Bgl*II cDNA fragment and a 537 bp *Bgl*II cDNA fragment. The digested pBluescript vector containing the 5' region of the human SHBG cDNA with *Stu*I and *Bgl*II ends, and the 537 bp *Bgl*II human SHBG cDNA fragment were both isolated by low melting agarose-gel electrophoresis (Wieslander, 1979). The analogous 306 bp rat ABP cDNA fragment was amplified from a cDNA by polymerase chain reaction (PCR) using a 5' oligonucleotide primer containing a *Stu*I site and a 3' primer containing a *Bgl*II site. The sequences of the oligonucleotide primers are: A) 5' GGGAGGCCTGAAATCCAGCTGCACAATCTC 3', corresponding to rat ABP cDNA nucleotides 352-375, the *Stu*I site is underlined; and B) 5' GGGAGATCTGGGCCTGGTGGCCCAGCCA 3', complimentary to rat ABP cDNA nucleotides 649-631; the *Bgl*II site is underlined. The PCR was performed in a 100 μL reaction mixture containing 1 μg plasmid DNA, 100 pmol of each primer, 250 μM of each dNTP and 2.5 units of *Taq* DNA polymerase, that was overlaid with mineral oil. The DNA template within the PCR mixture was initially denatured at 94°C for 4 min and then was subjected to 30 cycles of DNA amplification (template denaturation at 94°C for 2 min, primer annealing at 60°C for 2 min and primer extension at 72°C for 3 min). After the last cycle, the amplified DNA was heated to 72°C for 10 min to ensure complete extension of all PCR products.

The PCR products were digested with *Stu*I and *Bgl*II and the desired 306 bp rat ABP cDNA fragment was isolated by low melting agarose-gel electrophoresis (Wieslander, 1979) and directionally inserted immediately after the 5' region of the human SHBG cDNA contained within the isolated pBluescript vector. This vector now contained cDNA sequences for both human SHBG and rat ABP and was linearized with *Bgl*II to allow the previously isolated 537 bp *Bgl*II human SHBG cDNA fragment to be inserted in the correct orientation. This completed construction of the hybrid cDNA, which was then excised from pBluescript by *Hin*dIII and *Xba*I digestion and inserted into pRc/CMV for expression. The chimeric protein comprised amino acids 77-176 encoded by the rat ABP cDNA and

the remaining residues (1-76 and 177-373) encoded by the human SHBG cDNA (Figure 5.1).

2.2.5 Human SHBG cDNA Site-directed Mutants

The complete human SHBG cDNA (Figure 2.1, construct #6) was digested with *Hin*dIII and *Xba*I and inserted into a pSELECT vector, and single-stranded DNA was prepared for site-directed mutagenesis according to the 'Altered Sites' protocol provided by Promega. In brief, a vector-specific ampicillin-resistance repair primer and an appropriate SHBG mutagenic primer (Table 2.1) were both annealed to the single-stranded DNA template, and second strand DNA synthesis and ligation were achieved using T4 DNA polymerase and ligase, respectively. The DNA products of this reaction were used to transform a mismatch repair-deficient *E. coli* strain (BMH71-18 mut S) by the heat-shock method (Hanahan, 1985) and bacterial cells containing the pSELECT vector with a functional ß-lactamase (ampicillin resistance) gene were selected by growth in 125 μg ampicillin/mL SOC medium (2% Bacto-peptone, 0.5% Bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM glucose, 10 mM $MgCl_2$, pH 7.0). Plasmid DNA was isolated from the growing culture by the alkaline lysis method (Birnboim and Doly, 1979) and used to transform *E. coli* JM107. Plasmid DNA was isolated from individual ampicillin resistant colonies and sequenced (see 2.4) to identify clones with the appropriate mutation. The entire SHBG cDNA of each mutant cDNA clone was sequenced to confirm that only the targeted mutation had occurred, and these cDNAs were excised from pSELECT by *Hin*dIII and *Xba*I digestion, and inserted into pRc/CMV for expression in CHO cells.

2.3 Plasmid DNA Amplification and Purification

Small scale preparations of plasmid DNA were isolated from 1.5 mL cultures of bacteria (Birnboim and Doly, 1979), transformed with the appropriate vector. The yield of plasmid DNA (~ 5 μg) is suitable for DNA sequencing, diagnostic restriction enzyme analysis or isolation of cDNA fragments for sub-cloning. In addition, large scale cultures of bacteria (200 mL) containing expression vector

Table 2.1 A list of the oligonucleotide primers utilized for site-directed mutagenesis of the human SHBG coding region and the targeted amino acid substitution. Nucleotide(s) in the primer sequence that result in a mismatch with the single-stranded human SHBG cDNA template are in ***boldface*** p:int and *underlined*. Primer #31 was used to create a *Bam*HI site immediately 5' to the region coding for the mature SHBG polypeptide.

PRIMER #	TARGETED MUTATION	PRIMER SEQUENCE
1	Thr^{7} → Ala	5' ACTCTGGGCGGGGAGAA 3'
2	Asn^{351} → Gln	5' ATCTCATGGCTTCTCTGCAGGGCCTGGTC 3'
3	Asn^{367} → Gln	5' AAGCGTCAGTGCCTTGGCCTGGGCTCTG 3'
4	Asp^{327} → Asn	5' GGAAGAGTTTTCTCCTGG 3'
5	Cys^{164} → Ser	5' GGCGCAGGGAGCCATCCA 3'
6	Cys^{188} → Ser	5' CTACATCAGAGCTTCTGAGG 3'
7	Cys^{333} → Ser	5' GCCATTCAGGGAAAAAGAGG 3'
8	Cys^{333} → Stop	5' GCCATTCAGTCAAAAAGAGG 3'
9	Cys^{361} → Ser	5' TCTGGGGGGAGCTGTGA 3'
10	Ser^{133} → Asp	5' CCCCTGACCGACAAACGCCATC 3'
11	Lys^{134} → His	5' GATGGGATGGCGGTGGCTGGTCAGGGG 3'
12	Arg^{135} → Leu	5' GATGGGATGGAGTTTGCTGGTCAG 3'
13	His^{136} → Gln	5' GCAAACGCCAACCCATCATG 3'
14	Pro^{137} → Ala	5' CCTCATGATGGCATGGCGTTTGCT 3'
15	Ile^{138} → Ser	5' AATCCTCATGCTGGGATGGCGTT 3'

PRIMER #	TARGETED MUTATION	PRIMER SEQUENCE
16	$Met^{139} \rightarrow Trp$	5' AAGCGCAATCCTCCAGATGGGATGGCGT 3'
17	$Arg^{140} \rightarrow Leu$	5' CCCAAGCGCAATCAGCATGATGGGATG 3'
18	$Ala^{142} \rightarrow Glu$	5' CCCCCCAAGCTCAATCCTCATGAT 3'
19	$Gly^{144} \rightarrow Ala$	5' GAGCAGCCCCGCAAGCGCAATCCT 3'
20	$Gly^{144} \rightarrow Glu$	5' GAGCAGCCCCTCAAGCGCAATCCT 3'
21	$Gly^{145} \rightarrow Glu$	5' GAAGAGCAGCTCCCCAAGCGCAAT 3'
22	$Leu^{146} \rightarrow Lys$	5' AGCGGGGAAGAGCTTCCCCCCAAGCGC 3'
22	$Leu^{146} \rightarrow Ile$	5' AGCGGGGAAGAGTATCCCCCCAAGCGC 3'
23	$Leu^{147} \rightarrow Lys$	5'GTTGGAAGCGGGGAACTTCAG CCCCCCAAGCGC 3'
24	$Phe^{148} \rightarrow Leu$	5' GGAAGCGGGGAGGAGCAGCCC 3'
25	$Glu^{176} \rightarrow Gln$	5' GATGCTGAGATCTGGGCCTGTTTGTC 3'
26	$Asn^{193} \rightarrow Gln$	5' GAAATATCCCGGGCTGTGATTCTACATCAC 3'
27	$Leu^{198} \rightarrow Phe$	5' AGTCCCTGGAGGGAAAAATATCCCGGGG 3'
28	$Leu^{275,277,279,281} \rightarrow Ile$	5' CCCTGGACATACTGATCTTGATCTGAA TAGGGATTCCCAAGACCAGGGG 3'
29	$Lys^{39} \rightarrow Leu$	5' GGAGGAGGAGGTTAGTGTGATCTTGGTG 3'
30	$Ser^{42} \rightarrow Leu$	5' CCTCAAAGGAGAGGGAGGTTTTTGTG 3'
31	Create BamHI site	5' ACAGGTCTCAGGGATCCTCCCT GGCGGGTGTG 3'

DNA were extracted and purified on QIAGEN TIP 500 columns to yield 0.5 - 1.0 mg plasmid DNA as determined by spectrophotometric analysis. This purified DNA was used primarily for the transfection of mammalian cell lines, nucleotide sequencing, and the isolation of cDNA fragments to be radiolabeled as probes.

2.4 Nucleotide Sequence Analysis

The various cDNAs used for expression were sequenced to verify their open reading frames, and to confirm that only the targeted mutations were introduced. Double-stranded DNA templates were prepared for sequencing from 1-2 μg expression vector containing the cDNA, according to the instructions provided with a T7 Sequencing Kit (Pharmacia). Briefly, the DNA was denatured with 0.4 M sodium hydroxide, neutralized with 0.45 M sodium acetate and precipitated in 70% ethanol. The purified templates were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) using [α-^{35}S]dATP as the labeled nucleotide and reagents supplied by Pharmacia. Primers used for sequencing were supplied by Pharmacia (plasmid specific-M13 universal primer) or synthesized by the Molecular Biology Core Facility (SHBG cDNA-specific primers, plasmid-specific T7 and SP6 promoter primers). Chain elongation-termination reaction products were resolved by 8% polyacrylamide gel electrophoresis (PAGE) in the presence of 7 M urea. The gels were fixed in 15% methanol/10% glacial acetic acid (v/v) solution and vacuum-dried prior to autoradiography at room temperature using Kodak X-OMAT AR 5 film.

2.5 Expression of SHBG cDNAs in Mammalian Cells

2.5.1 Expression Vectors

The pRc/CMV vector that contains the cytomegalovirus promoter and enhancer and the gene conferring neomycin resistance, was utilized for the stable, constitutive expression of the human SHBG cDNA, the human SHBG/rat ABP hybrid cDNAs, and the human SHBG mutant cDNAs, in mammalian cells. The pBPV vector contains the entire bovine papilloma virus genome for stable expression of the human SHBG cDNA in CHO cells without integration of the plasmid into the host

genome. This vector utilizes the Moloney murine sarcoma virus enhancer and requires a heavy metal, such as 10 μM $CdCl_2$, to induce expression of SHBG from the mouse metallothionein-I promoter, however it does not contain a selectable growth marker. Therefore, the pBPV construct was co-transfected with pSV2neo, a plasmid that contains the gene that confers neomycin resistance. All plasmid DNA expression constructs were propagated in large scale cultures of *E. coli* JM107 and purified using QIAGEN columns (see 2.3) for transfection into mammalian cells.

2.5.2 Transfection and Selection of Stable Transformants

The various expression constructs were transfected into CHO cells using the polybrene/dimethylsulphoxide technique (Chaney *et al.*, 1986). In brief, exponentially growing CHO cells (5 x 10^5) were incubated overnight in MEM/FBS, the medium was replaced with a minimal volume (3-4 mL) of MEM/FBS containing the pRc/CMV expression construct DNA (5-20 μg) or pBPV expression construct DNA/pSV2neo (10 μg/1 μg), followed by 5 μL of polybrene (Aldrich) solution (10 mg/mL sterile water). The flasks were agitated several times for 6 h at 37°C, after which the DNA/polybrene mixture was removed, and 5 mL 30% dimethylsulphoxide (DMSO) in MEM/FBS was added for 4 min. The cells were then immediately washed with PBS to remove the DMSO solution.

The BW-1 cells ($1x10^7$) were harvested at late log phase and transfected with the pRc/CMV vector containing the SHBG cDNA (6.5-13 μg) in 800 μL phosphate-buffered saline (PBS), by electroporation using a Bio-Rad Gene Pulser™ in a 0.4 cm cuvette at 0.4 kVolts and 250 μFarads.

Transfected cells were cultured in 25 mL MEM/FBS for 48 h to allow for recovery prior to the addition of 1.8 mg G418/mL for selection of neomycin resistant cells. After 10-12 days, G418 resistant colonies were harvested and reseeded cells were grown to confluency in the presence of G418 for an additional 10 days. Transfected cells were conditioned to Dulbecco's modified Eagle medium (DMEM)

containing 10 mM HEPES, penicillin (5 units/mL), streptomycin (5 mg/mL) with or without 2% Ultroser G (DMEM/UltroSer G), a serum-substitute.

2.6 Total RNA Extraction From Mammalian Cell Lines

Total RNA was extracted from both wild-type CHO and BW-1 cells, and cells containing a human SHBG cDNA expression construct, using the LiCl/Urea technique (Auffray and Rougeon, 1980). Briefly, confluent cell cultures in T-150 flasks (5 x 10^7 cells) were harvested by treatment with 0.25% trypsin/1 mM EDTA and centrifuged. Cell pellets were snap-frozen with liquid nitrogen and stored at -80°C until needed. Frozen cell pellets were resuspended in 3 M LiCl/6 M urea containing 50 units heparin/mL, vortexed and incubated overnight at 4°C. The sample was then centrifuged at 31,000 x g for 30 min and the pellet was resuspended in the same solution and centrifuged again. The pellets were resuspended in 10 mM Tris-HCl, pH 7.5, containing 0.5% sodium dodecylsulphate (SDS) and 100 units heparin/mL, and the RNA was extracted with chloroform/isoamyl alcohol (24:1) prior to ethanol precipitation. After two washes with 70% ethanol, total RNA was resuspended in diethylpyrocarbonate-treated sterile water containing 70% ethanol and stored at -80°C. An aliquot of the RNA was measured for purity and quantity by spectrophotometric analysis. To assess the quality and relative quantity of isolated RNA, an aliquot of each sample (4.5 μg) was mixed in a final concentration of 0.5 x running buffer [20 mM 3-(N-Morpholino)propanesulphonic acid (MOPS), 5.3 mM sodium acetate, 0.53 mM EDTA, pH 7.0], 2 M formaldehyde and 50% deionized formamide (v/v). The RNA samples were denatured at 50°C for 30 min and rapidly cooled for 10 min in ice-water. The samples were then subjected to electrophoresis in a 1.1% agarose gel containing 1 x running buffer (40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, pH 7.0) and 2.2 M formaldehyde (Rave *et al.*, 1979). After staining with a solution of 10 μg ethidium bromide/100 mL containing 125 μL ß-mercaptoethanol, intact RNA was assessed by the integrity of the 28S and 18S ribosomal RNA bands.

2.7 Northern Blot Analysis

Ten μg of total RNA was resolved by electrophoresis (see 2.6) and was transferred to a Zeta-probe nylon membrane (Bio-Rad Laboratories) by capillary blotting (Thomas, 1980) and cross-linked by exposure to UV light for 10 min. The blot was baked in a vacuum oven (2 h at 80°C) and then incubated in ZetaProbe prehybridization solution [4 x SSPE (600 mM NaCl, 40 mM $NaH_2PO_4 \bullet 2H_2O$, 4 mM EDTA, pH 7.4), 1% SDS, 0.5% skim milk powder, and 0.5 mg sheared denatured salmon sperm DNA/mL] at 42°C for 5 hrs. A 555 bp human SHBG cDNA fragment (Hammond *et al.*, 1987) was labeled with [α-^{32}P]dCTP by random hexanucleotide priming and Klenow enzyme (Feinberg and Vogelstein, 1983) and unincorporated radiolabel was removed by gel filtration using a Sephadex G-50 NICK column (Pharmacia). The blot was probed overnight at 42°C and then washed to high stringency in 0.2 x SSC (30 mM NaCl, 3 mM tri-sodium citrate, pH 7.0), 0.1% SDS at 42°C, prior to autoradiography for 48 h at -80°C.

To control for the amount of RNA loaded on the gel and transferred to the membrane, the human SHBG cDNA probe was removed from the blot by washing the membrane five times in a boiling solution of 0.1 x SSC (15 mM NaCl, 1.5 mM tri-sodium citrate, pH 7.0), 0.1% SDS, for 5 min each. The membrane was then blocked in prehybridization solution and re-probed with a ^{32}P-labeled mouse 18S ribosome cDNA at 42°C, prior to autoradiography for 4 h at -80°C.

2.8 Glutathione S-Transferase/Human SHBG Fusion Proteins

2.8.1 Human SHBG cDNA Deletion Mutants

A human SHBG cDNA in the pSELECT vector (see 2.2.5) was mutated using a mutagenic oligonucleotide primer (Table 2.1) to introduce a *Bam*HI site immediately 5' to the codon for Leu^1 in the mature polypeptide (Hammond *et al.*, 1987). Since another *Bam*HI site was located 3' of the coding region, the mutated SHBG cDNA was excised from the pSELECT vector by *Bam*HI digestion and sub-cloned into a *Bam*HI-digested pGEX-2T vector (Pharmacia). This places the SHBG cDNA downstream and in frame with the glutathione S-transferase (GST)

gene, and the resulting construct encodes a GST/SHBG fusion protein, designated SHBG(1-373) as shown in Table 6.4. Digestion with either *Eco*RI, *Sma*I, or *Bgl*II and *Eco*RI, selectively removed a 3' portion of the human SHBG cDNA, and created cDNA constructs that encoded GST fusion proteins containing either the *N*-terminal 205, 194 or 177 amino acids of SHBG, respectively [see SHBG(1-205), SHBG(1-194) and SHBG(1-177) in Table 6.4].

2.8.2 Expression of Fusion Proteins in *E. coli*

The different pGEX-2T constructs were used to transform the *E. coli* JM107 strain in order to express the GST fusion proteins and the original protocol (Smith and Johnson, 1988) was modified as follows: Bacterial cultures (250 mL) were grown for approximately 4 h at 37°C (optical density at 600 nm = 0.7-1.0), and then cooled for 15 min at room temperature prior to the addition of 0.5 mM isopropyl-ß-D-thiogalactoside (Promega). After further incubation for 90 min at 29°C, the cultures were centrifuged (5,000 x g for 10 min at 4°C) and the bacterial pellets were resuspended in 50 mL PBS. Lysozyme (Sigma) was added to a concentration of 1 mg/mL prior to incubation on ice for 20 min. Triton X-100 (BDH) was then added to a final concentration of 1%, and the suspensions were incubated on ice for 10 min before ultrasonic disruption. The sonicated suspensions were centrifuged for 1 h at 9,000 x 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). After washing the columns with PBS, the bound protein was eluted with 50 mM Tris-HCl, pH 8.0, containing 10 mM reduced glutathione (GIBCO BRL). Fractions containing eluted GST fusion proteins were pooled, NaCl was added to a final concentration of 3 M, and free glutathione was removed by gel filtration using a 3 mL Sephadex G-25 column (Pharmacia).

2.9 Immunochemical Detection of SHBG

2.9.1 SHBG Immunoradiometric Assay

Human SHBG concentrations were measured using an immunoradiometric

assay (IRMA) that utilizes a mouse monoclonal IgG antibody against human SHBG (Hammond *et al.*, 1985; Hammond and Robinson, 1984). Aliquots of samples (100 μL) were first incubated with 100 μL ^{125}I-labeled mouse monoclonal antibody for 30 min at room temperature and then mixed with a rabbit polyclonal antiserum against human SHBG (100 μL) and incubated for a further 60 min. A donkey anti-rabbit IgG antibody covalently linked to kaolin as solid-phase (Orion Diagnostica) was added to the samples and vortexed, followed 30 min later with 2 mL 0.9% NaCl. After centrifugation (2000 x g for 10 min) to pellet the solid-phase antibody/SHBG complexes, the supernatants were aspirated and the radioactivity in the pellets was measured using a gamma counter.

2.9.2 SHBG Radioimmunoassay

Human SHBG purified from serum (25 μg) and stored in 0.1 M borate buffer (pH 8.0), was added to evaporated [^{125}I]Bolton-Hunter reagent and incubated at 4°C for 16 h. Radiolabeled SHBG was separated from unincorporated radiolabel and low molecular weight material by gel filtration using a Sephadex G-25 column (PD-10, Pharmacia), which was pre-equilibrated and eluted with 0.1 M Tris-HCl,pH 8.0, 0.1 M NaCl, 0.3% gelatin. Labeled SHBG had a specific activity of 2.3 μCi/μg protein.

Recombinant SHBGs were assessed for their ability to compete with [^{125}I]SHBG for a rabbit polyclonal antiserum against human SHBG. Samples containing 0.1-7.0 pmol SHBG/mL, as determined by steroid-binding capacity assay (see 2.11.1), were incubated overnight at room temperature with 100 μL of a rabbit polyclonal antiserum against human SHBG (diluted 1:50) and 100 μL [^{125}I]SHBG (15,000 cpm). Kaolin-linked donkey anti-rabbit antibody (500 μL) was then added and incubated for 1 h at room temperature. Following centrifugation (2,000 x g, 10 min), supernatants were aspirated and the radioactivity in the pellets was measured using a gamma counter.

2.9.3 SHBG Homodimer Assay

The mouse monoclonal antibody utilized in the IRMA (see 2.9.1) was used to demonstrate that SHBG contains two identical epitopes per dimer, and exists as a homodimer in solution (Hammond *et al.*, 1986). This was done by using the mouse monoclonal antibody as both immobilizing and radiolabeling reagents in an immunoassay (Hammond and Robinson, 1984). To immobilize the antibody, it was conjugated to cellulose (Sigmacell Type 20) activated with 1,1'-carbonyl-diimidazole (Sigma) (Chapman and Ratcliffe, 1982). A separate batch of monoclonal antibody was labeled with [^{125}I]Bolton-Hunter reagent, according to the instructions provided by Dupont NEN.

Assay tubes contained 100 μL [^{125}I] mouse monoclonal antibody (40,000 cpm), 100 μL of mouse monoclonal antibody-cellulose (20 mg/mL), and 100 μL human serum or culture medium, diluted serially in MEM/FBS to yield human SHBG concentrations between 2.0 - 0.06 pmol/mL. Non-specific background was measured in tubes containing 100 μL MEM/FBS in place of human SHBG samples. After 16 h at room temperature, 2 mL 0.9% NaCl was added to the assay tubes which were then centrifuged (15 min at 2,500 x g). Supernatants were aspirated and the radioactivity in the pellets was measured using a gamma counter.

2.10 Affinity Chromatography

2.10.1 Blue-Sepharose Chromatography

Albumin binds steroids with low affinity and is a major component of UltroSer G, which is used as a serum substitute in tissue culture medium. Therefore samples of human SHBG in DMEM containing 2% UltroSer G were applied to a Blue-Sepharose column to remove albumin from the culture medium prior to steroid-binding analyses. The samples of SHBG in DMEM only did not require this treatment before steroid-binding analysis.

Culture medium containing UltroSer G was collected and dialyzed against 50 mM Tris-HCl, pH 7.5, for 36 h at 4°C to remove salt, and 10 mL samples were

applied to 6 mL reactive Blue 2-Sepharose CL 4B (Sigma) columns, pre-equilibrated with 50 mM Tris-HCl, pH 7.5. Fractions (1 mL) containing protein that did not bind to the column were collected, and immunoreactive SHBG was detected (see 2.9.1) in fractions 4-15, which were pooled for steroid-binding analyses, while the majority of albumin remained bound to the column. This resulted in an approximately 20-fold purification of SHBG due largely to the removal of contaminating albumin.

2.10.2 Concanavalin A Chromatography

In order to resolve SHBG into different isoforms based on variations in glycosylation, culture medium and diluted serum samples containing SHBG were applied under gravity to 5 mL concanavalin (Con) A-agarose (Type III-AS, Sigma) columns, pre-equilibrated in Con A buffer (50 mM Tris, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.4). Fractions (1 mL) were collected until unbound protein was removed, after which Con A buffer containing 250 mM methyl-α-D-mannopyranoside was applied and 1 mL fractions of eluted protein were collected. An IRMA (see 2.9.1) was used to identify fractions containing SHBG.

2.11 Steroid-binding Capacity Assay

2.11.1 Saturation Analysis

The steroid-binding capacity of samples containing SHBG was determined by saturation analysis using [^{3}H]DHT as labeled ligand, and a slurry of dextran-coated charcoal (DCC) in PBS to separate bound and free steroid (Hammond and Lähteenmäki, 1983). Briefly, endogenous steroids were removed from serum samples by dilution (1:200) in a DCC suspension [0.25% Norit A neutral charcoal (BDH), 0.025% Dextran T70 (Pharmacia), in 0.1 X PBS containing 0.01% gelatin (Sigma) and 0.01% sodium azide (JT Baker Chemicals)] and incubating for 30 min at room temperature. Culture medium samples were mixed with a pellet of DCC to avoid sample dilution, and then incubated in the same way. After centrifugation (3,000 x g for 10 min), the culture medium supernatants were diluted with DMEM

if required. Aliquots (100 μL) of steroid-free serum or culture medium samples were incubated with 10 nM [^{3}H]DHT (42 or 48 Ci/mmol), in the presence (nonspecific background binding) or absence (total binding) of 2 μM unlabeled DHT for at least 1 h at room temperature followed by 30 min at 0°C. A 600 μL slurry of DCC was then added to the samples and incubated for another 10 min at 0°C to adsorb any unbound steroid from solution. After centrifugation (3,000 x g for 10 min at 0°C), supernatants were decanted into counting vials and mixed with 4 mL Aqueous Counting Scintillant (Amersham), prior to measurement of radioactivity using a scintillation spectrophotometer.

2.11.2 Steroid 'Off-rate' Analysis

The apparent dissociation rate or 'off-rate' of [^{3}H]DHT from the steroid-binding sites of various forms of SHBG was determined by preparing samples as in 2.11.1, except that the amount of protein-bound [^{3}H]DHT was measured after the samples were incubated with 600 μL DCC for increasing lengths of time (1-15 min) at 0°C. The amount of protein-bound [^{3}H]DHT at time=0 was estimated by extrapolation of the data graphed as counts per minute (cpm) versus time. The 'off-rate' is interpolated from the graph as the percent difference between the amount of protein-bound [^{3}H]DHT after 10 min of DCC exposure relative to the amount of protein-bound [^{3}H]DHT at time=0. This assay is a measure of the dissociation of the labeled ligand from the binding protein and the 'off-rate' of [^{3}H]DHT from human SHBG in serum is < 5% after 10 min DCC exposure.

2.11.3 Scatchard Analysis

The affinities of various forms of SHBG for [^{3}H]DHT were determined by Scatchard analysis (Scatchard, 1949) and expressed as dissociation constants (k_d). Samples were treated with DCC to remove endogenous steroid and analyzed as in 2.11.1, except that the samples were incubated with increasing amounts of [^{3}H]DHT (1,000-30,000 cpm) with or without 2 μM unlabeled DHT. Protein-bound and free DHT were separated by DCC treatment for 10 min at 0°C, prior to quantification of radioactivity.

2.11.4 Competition Analysis

The relative binding affinities of various forms of SHBG for other steroid ligands were determined using [^{3}H]DHT as the labeled ligand, and incubations were performed in the presence of increasing amounts of unlabeled DHT, T, E_2, progesterone (P), cortisol (F), 19-nor-testosterone (19norT), 5α-androstane-3ß,17ß-diol (5αA) and andro-5-stene-3ß,17ß-diol (Δ5A) as competitors. Protein-bound and free steroid were separated by DCC treatment for 10 min at 0°C, prior to quantification of radioactivity. The relative binding affinity of SHBG for each steroid was determined as the amount of unlabeled DHT required to displace 50% of the labeled ligand, divided by the amount of other competitors required to displace 50% of the labeled ligand.

2.12 Gel Filtration Chromatography

The molecular sizes of natural and recombinant SHBG samples were determined by subjecting 100 μL samples of serum from an individual (diluted 1:5) or culture medium (concentrated 10-fold), to gel filtration on a Superose 6 FPLC column (Pharmacia LKB Biotechnology). Chromatographic fractions were monitored for SHBG content by the IRMA (Hammond *et al.*, 1985), and the molecular sizes were calculated by comparison with the elution volumes of the following molecular size (M_r) standards: bovine thyroglobulin (M_r=670,000); bovine gamma globulin (M_r=158,000); chicken ovalbumin (M_r=44,000); horse myoglobin (M_r=17,000) and cyanocobalamin (M_r=1,350).

2.13 Immunopurification of SHBG

A mouse monoclonal antibody specific for human SHBG (Hammond and Robinson, 1984; Hammond *et al.*, 1985) was immobilized on microparticulate cellulose (see 2.9.3) and this was used to immunopurify and concentrate SHBG from serum or culture medium. Samples (1-5 mL) that contain SHBG were mixed with a 100 μL slurry of immobilized monoclonal antibody and rotated for 6 h at room temperature. After centrifugation and removal of the supernatants, the monoclonal antibody-cellulose pellets were washed with 1 mL PBS and then

resuspended in 50 μL PBS and 25 μL denaturing-PAGE load buffer (2% SDS, 5% ß-mercaptoethanol, 1% glycerol, 60 mM Tris-HCl, pH 6.8, bromophenol blue crystals).

2.14 Western Blot Analysis

Various protein samples were subjected to discontinuous electrophoresis in a 4% acrylamide stacking gel and 7.5% or 10% acrylamide resolving gels. For denaturing- PAGE in the presence of SDS, immunopurified SHBG bound to the monoclonal antibody-cellulose (see 2.13) was resuspended by vortexing and dissociated from the antibody by heating at 90°C for 2 min. The cellulose was sedimented and aliquots (3-15 μL) of the supernatants containing SHBG were loaded on the gel. Aliquots (5-20 μL) of SHBG from diluted serum (1:200) or culture medium, that were not immunopurified, were mixed with 0.5 vol of denaturing load buffer, prior to heating and loading on the gel. For nondenaturing-PAGE, aliquots of SHBG from diluted serum (1:200) or culture medium were mixed with load buffer lacking SDS and ß-mercaptoethanol, prior to loading on the gel. Prestained SDS-PAGE molecular size markers (Bio Rad) were included in gels and electrophoresis was performed at 15 volts/cm.

Proteins were electro-transferred from the gel onto Immobilon-P PVDF membranes (Millipore) for 1.5 h at 300 mAmps. The membranes were blocked with 4% bovine serum albumin (BSA) and 0.05% Tween 20 (v/v) in 10 mL TBS (50 mM Tris pH 8.0, 150 mM NaCl) for at least 30 min at room temperature. The blots were then incubated (16 h at 4°C) with a rabbit polyclonal antiserum against human SHBG (Hammond *et al.*, 1985) diluted 1:500 in 5 mL TBS containing 1% BSA and 0.05% Tween 20. After washing with TBS, the membranes were incubated with an alkaline phosphatase-labeled goat anti-rabbit IgG secondary antibody (1:5000 in TBS) for 5 h at room temp, and immunoreactive proteins were visualized in a solution containing 700 μM 5-bromo-4-chloro-3-indolyl phosphate, 200 μM nitro blue tetrazolium, 1 mM levamisole (Sigma) and alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

2.15 Expression of Human SHBG in Nude Mice

Exponentially growing CHO cells expressing human SHBG were routinely cultured in MEM/FBS and harvested using trypsin/EDTA from T75 flasks to yield approximately 5 x 10^6 cells. The cells were immediately centrifuged for 2 min at 3,000 x g, the medium was aspirated and the cell pellet was resuspended in 1 mL of MEM/FBS. Adult male nude mice were inoculated subcutaneously with 0.5 mL of the cell suspension in the haunch above one of the hind legs. After 10 days, a mass of cells was evident at the site of injection, and the tip of the tail was cut to collect 500 μL of blood. After the blood was allowed to clot at room temperature, the amount of human SHBG in the serum was measured by IRMA. Furthermore, 100 μL of serum was withdrawn and diluted to 1 mL in Con A buffer and subjected to Con A chromatography (see 2.10.2). Immunoreactive human SHBG was detected in the collected fractions by an IRMA (see 2.9.1).

CHAPTER 3

EXPRESSION OF RECOMBINANT HUMAN SHBG IN MAMMALIAN CELLS

3.1 Introduction

To study the structure and biosynthesis of human SHBG, it was necessary to create an *in vitro* expression system that would generate sufficient quantities of recombinant SHBG for analyses. The first step in developing this system required the construction of a cDNA encoding for the human SHBG precursor polypeptide, which would allow expression and secretion from a eukaryotic expression vector in mammalian cells. In addition, it was important that the recombinant product was structurally and functionally equivalent to natural SHBG, and an efficient system needed to be identified and characterized with respect to the most suitable expression vector, host cell line, method of transformation, and tissue culture conditions.

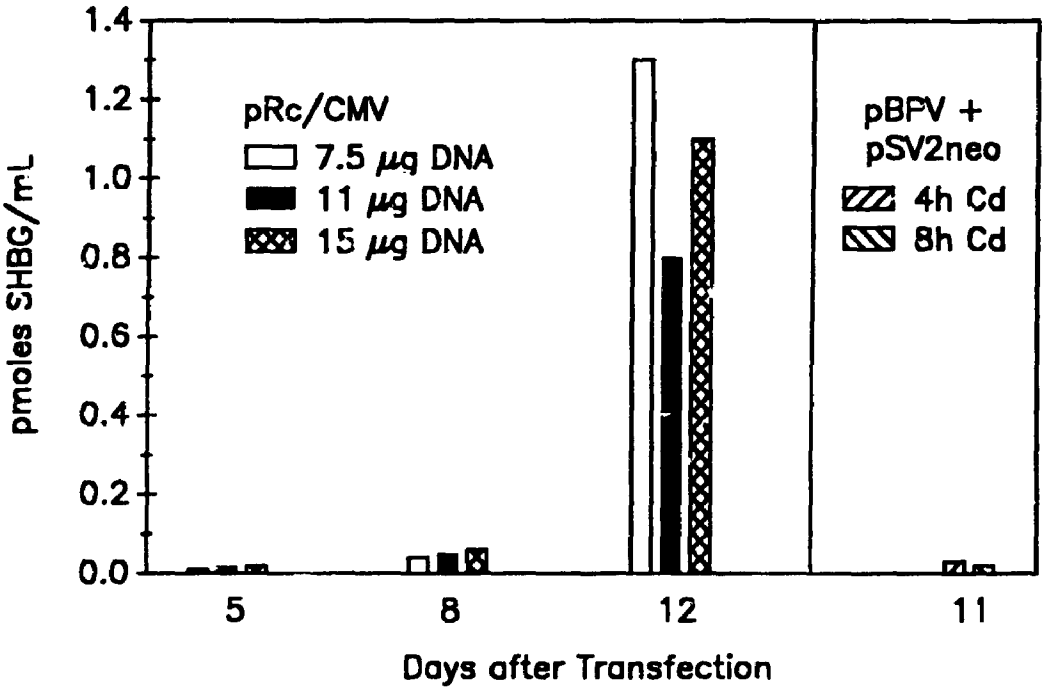
My initial studies involving the production of recombinant SHBG also addressed some unresolved issues concerning its synthesis. These included the definition of cell-specific post-translational modifications of SHBG, the carbohydrate composition of SHBG subunits, and the basis for subunit-size heterogeneity.

3.2 Results

3.2.1 Expression of Recombinant Human SHBG

To generate recombinant SHBG in quantities sufficient for analysis, it was necessary to test several eukaryotic expression systems. A human SHBG cDNA contained within a 1403 bp *Not*I fragment (Figure 2.1, construct #5) was inserted into the cloning sites of both the pBPV and pRc/CMV eukaryotic expression vectors and these constructs were transfected into CHO cells (see 2.2.1). Eleven days after transfection, the pBPV vector produced minute quantities of SHBG (0.03 nM) after induction with 10 μM $CdCl_2$ for 4 or 8 h (Figure 3.1). If the cells were exposed to $CdCl_2$ for longer than 8 h or to concentrations greater than 10 μM, greater than 90% of the cells lost viability within a day. Conversely, the pRc/CMV vector produced 45-fold more SHBG (1.3 nM) than pBPV, twelve days after transfection. Furthermore, it required no toxic agents to induce SHBG production and SHBG was constitutively produced by these cells for longer than 60 days. The pRc/CMV

Figure 3.1 Comparison of the production of recombinant human SHBG from CHO cells transfected with two different expression constructs. Human SHBG secreted into the culture medium was detected by an IRMA at the indicated times after transfection. The cells were transfected with the indicated amounts of the pRc/CMV construct. The cells transfected with pBPV (10 μg) and pSV2neo (1 μg) were subjected to 10 μM $CdCl_2$ treatment (*Cd*) for the indicated time before analysis.


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
pmoles SHBG/mL
pRc/CMV
7.5 μg DNA
11 μg DNA
15 μg DNA
pBPV +
pSV2neo
4h Cd
8h Cd
5
8
12
11
Days after Transfection

vector was selected for the expression of recombinant human SHBG in mammalian cells.

The biosynthesis of SHBG was studied in a CHO cell line that is frequently used as an expression host for mammalian proteins (Chaney *et al.*, 1986), and in murine BW-1 hepatoma cells (Szpirer and Szpirer, 1975). This was done to determine which cell line was most effective for the production of human SHBG and to examine if there was a qualitative difference in recombinant proteins produced by these cells. During the course of defining the most effective expression system, it was found that transfection using polybrene/DMSO was a very efficient way of introducing the expression vector into CHO cells, while electroporation resulted in the death of > 90% of these cells within 24 h. By contrast, electroporation was a much more effective means of transfecting DNA into BW-1 cells (see 2.5.2), when compared to the polybrene/DMSO technique.

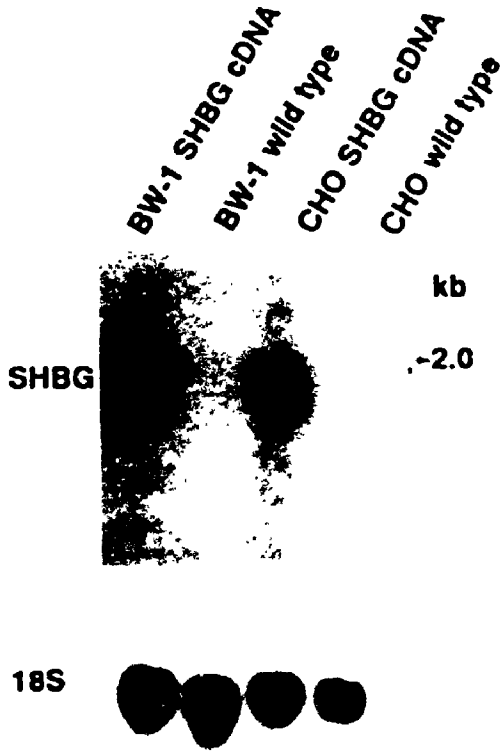
3.2.2 Northern Analysis of Human SHBG mRNA

Both cell lines were transfected with pRc/CMV containing the human SHBG *Not*I cDNA fragment (Figure 2.1, construct #5), and contained an SHBG mRNA of approximately 1.9 kb in size (Figure 3.2). The abundance of the SHBG mRNA was assessed with respect to the 18S ribosomal RNA content of cellular extracts, and densitometric analyses indicated that CHO cells contain approximately four times more SHBG mRNA than the BW-1 cells (Figure 3.2). The specificity of this observation was confirmed by the lack of any cross-hybridizing mRNA species in untransfected CHO or BW-1 cells (Figure 3.2).

3.2.3 Steroid-binding Properties of Recombinant Human SHBG

Prior to the collection of culture medium for analyses, the cells were conditioned to serum-free medium because FBS contains bovine SHBG which interferes with the steroid-binding measurements of recombinant human SHBG. However, in our preliminary experiments the high concentration of albumin in the serum substitute used in the culture medium (2% UltroSer G) also affected the

Figure 3.2 Northern blot of total RNA isolated from untransfected wild-type and SHBG cDNA-transfected CHO and BW-1 cells. Total RNA (10 μg) was subjected to 1.1% agarose gel electrophoresis in the presence of formaldehyde, transferred to a Zeta-probe membrane and hybridized with a 555 bp [^{32}P]human SHBG cDNA and a [^{32}P]mouse 18S ribosomal cDNA. The hybridizing RNA species are identified on the *left* and a size marker is indicated to the *right*.


BW-1 SHBG cDNA
BW-1 wild type
CHO SHBG cDNA
CHO wild type
kb
~2.0
SHBG
18S

steroid-binding assays, and the albumin had to be essentially removed by Blue-Sepharose affinity chromatography before analysis (see 2.10.1). Although the culture medium harvested from untransfected cell lines did not contain any SHBG-like steroid-binding activity, BW-1 cells secrete high levels of both albumin and α-fetoprotein (Szpirer and Szpirer, 1975). Mouse α-fetoprotein is known to have a relatively high affinity for estrogens (Savu *et al.*, 1981). Therefore, the use of BW-1 cells was abandoned to avoid any possible interference from these murine proteins on the subsequent studies of the steroid-binding properties of recombinant SHBG.

When SHBG produced by CHO cells was examined by Scatchard analysis (Scatchard, 1949), its affinity for DHT was found to be essentially identical to SHBG in diluted serum; the dissociation constant (k_d) was 0.65 nM for the recombinant SHBG and 0.67 nM for serum SHBG (Figure 3.3). Furthermore, the steroid-binding specificities of natural and recombinant SHBG were very similar (Table 3.1).

3.2.4 Immunochemical Analyses of Recombinant Human SHBG

The immunochemical properties of SHBG produced by CHO and BW-1 cells were assessed using a mouse monoclonal antibody against human SHBG (Hammond and Robinson, 1984) in an assay (Hammond *et al.*, 1986) in which only SHBG dimers containing two identical epitopes precipitate as a radiolabeled complex (see 2.9.3). The dose-response curves generated for SHBG in culture medium were essentially identical to that obtained for serum SHBG, and demonstrate that the recombinant product from both cell types forms a homodimer (Figure 3.4).

The amount of SHBG in the culture medium from transfected CHO and BW-1 cells was measured by IRMA (see 2.9.1) and steroid-binding capacity assay (see 2.11.1). As in the case for serum SHBG, the levels of recombinant SHBG measured by both assays were equivalent, which indicates that the immunological and steroid-binding properties of the recombinant protein are very similar to its

Figure 3.3 Scatchard plots of SHBG in CHO cell culture medium and human serum. The dissociation constants (k_d) were measured at 0°C using [^{3}H]DHT as the labeled ligand and DCC tc separate bound and free steroid. Each *data point* represents the mean value of triplicate measurements. Wild-type recombinant SHBG in medium, k_d=0.65 nM; SHBG in human serum, k_d=0.67 nM.

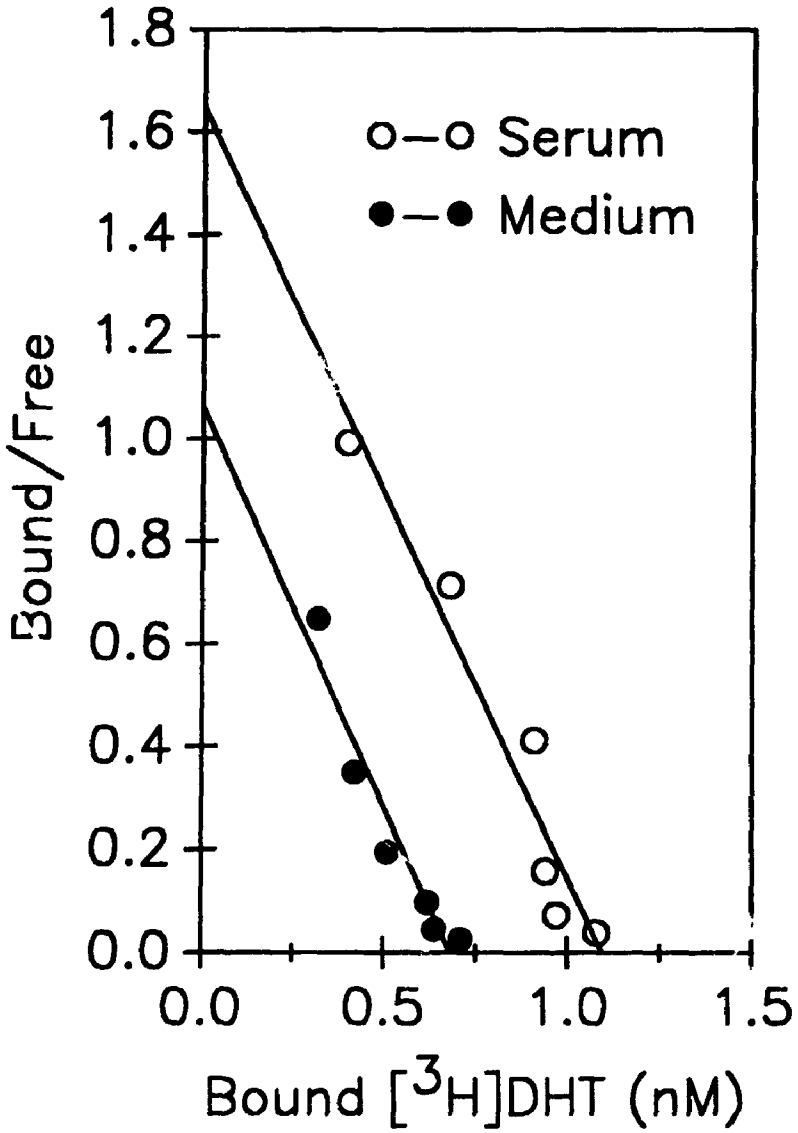
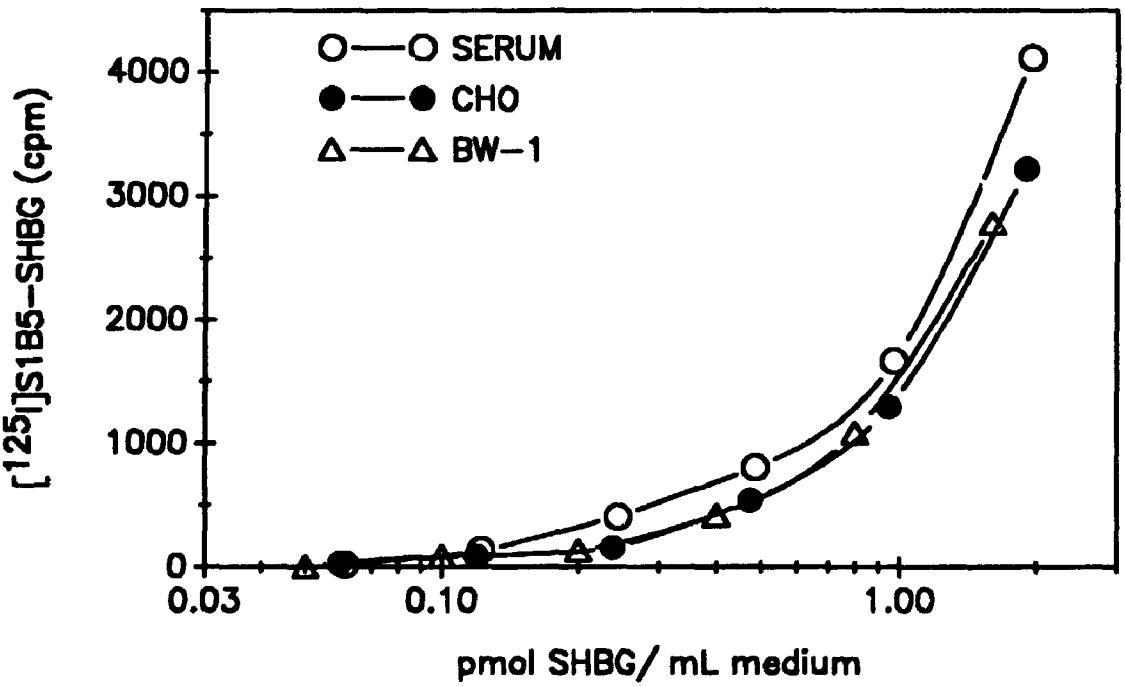

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Bound/Free
o—o Serum
●—● Medium
0.0
0.5
1.0
1.5
Bound [3H]DHT (nM)

Table 3.1 The relative binding affinities of natural and recombinant human SHBG for different steroids. The relative binding affinity of SHBG for each steroid was determined as the amount of DHT required to displace 50% of the labeled ligand, divided by the amount of other competitor steroids to displace 50% of the labeled ligand. Measurements were taken at 0°C using [^{3}H]DHT as the labeled ligand and DCC to separate bound and free steroid.

STEROID	RELATIVE BINDING AFFINITY	
	NATURAL	RECOMBINANT
5α-Dihydrotestosterone	100.00	100.00
Testosterone	19.67	25.00
17ß-Estradiol	6.86	6.67
Progesterone	0.08	0.17
Cortisol	<< 0.01	<< 0.01

Figure 3.4 Immunochemical detection of recombinant and serum SHBG based on two subunits with an identical epitope. Serial dilutions of culture medium and serum containing human SHBG were incubated with an ^{125}I-labeled monoclonal antibody specific for human SHBG, in the presence of the same unlabeled antibody coupled to cellulose (see 2.9.3). After centrifugation, the pellets containing [^{125}I]antibody/SHBG antibody-cellulose complexes were quantified for radioactivity. Each *data point* represents the mean value of triplicate measurements.


O—O SERUM
●—● CHO
Δ—Δ BW-1
4000
3000
2000
1000
0
0.03
0.10
1.00
[125I]S1B5-SHBG (cpm)
pmol SHBG/ mL medium

natural counterpart. In a separate experiment, when the levels of secreted SHBG were measured in medium taken from the same cells utilized for the isolation of RNA, the results showed that BW-1 cells produced twice as much SHBG (2 pmol/mL) compared to CHO cells (1 pmol/mL), despite the fact that the CHO cells contained 4-fold more SHBG mRNA.

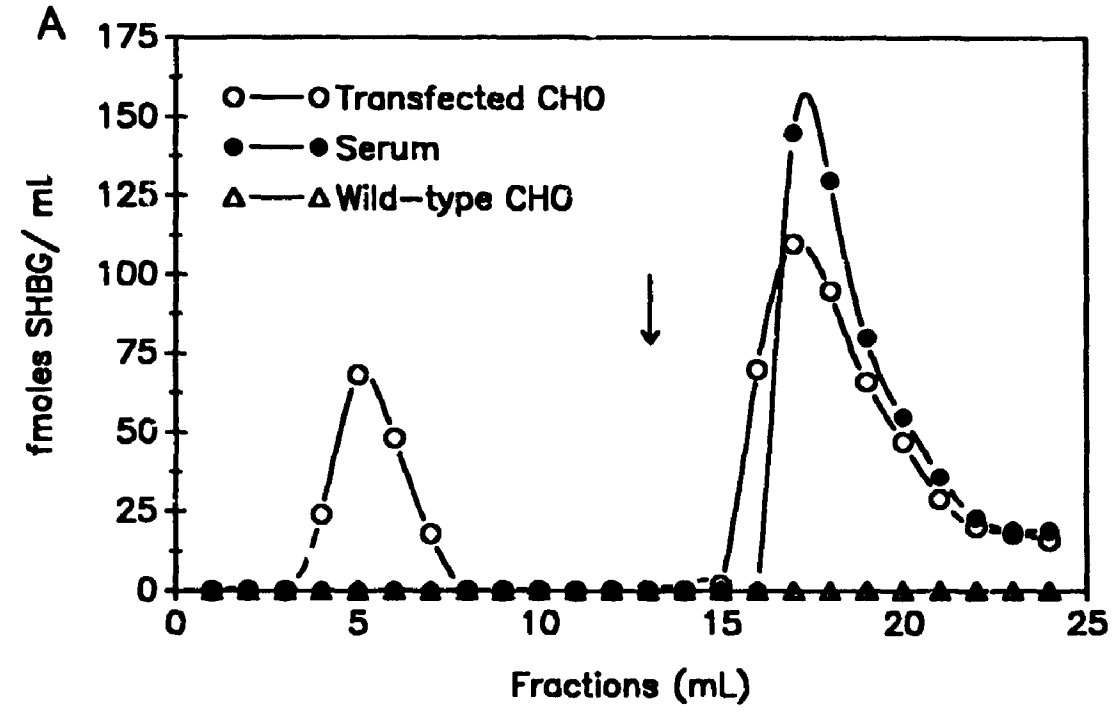
3.2.5 Concanavalin A-binding Properties of Recombinant Human SHBG

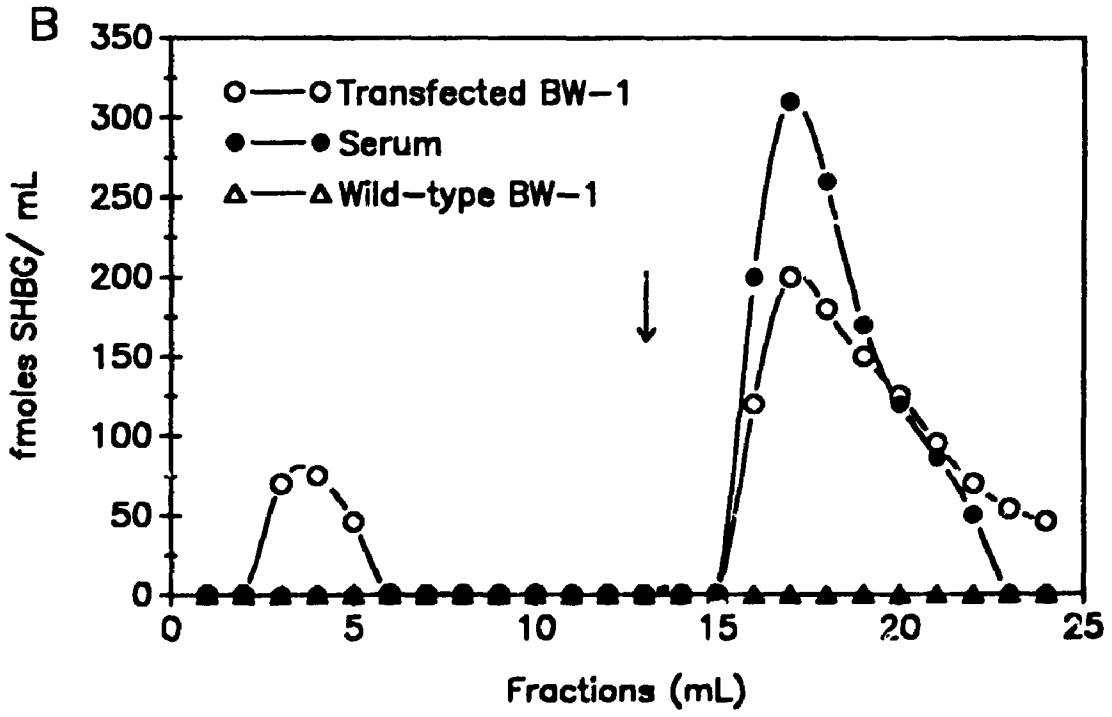
Concanavalin A (Con A) is a plant lectin that binds to proteins containing at least one *N*-linked biantennary or high mannose-type oligosaccharide chain, while proteins that contain more branched carbohydrates or completely lack *N*-linked oligosaccharides do not interact with this lectin (Krusius *et al.*, 1976; Narasimhan *et al.*, 1979). Therefore, samples of culture medium from transfected CHO and BW-1 cells were analyzed by Con A chromatography, and the elution profiles of immunoreactive SHBG were compared to those obtained for human serum and control medium from untransfected cells (Figure 3.5). Immunoreactive SHBG in serum and medium from both transfected cell types resolved into two forms; i.e. SHBG that did not bind to Con A (Con A^-), and SHBG that interacted with Con A and eluted in the presence of mannopyranoside (Con A^+). Only a very small proportion (<2%) of the SHBG in serum did not interact with Con A, while a much larger proportion of the SHBG produced by both cell types was present in the Con A^- fraction. Furthermore, the ratio of immunoreactive SHBG in these two fractions differed between the cell types, and the percentages (mean ± SD) of SHBG that bound to Con A were 73.8 ± 1.2 % and 86.0 ± 1.3 % for the CHO and BW-1 cells, respectively, when medium samples from three separate cultures of each cell type were examined.

3.2.6 Electrophoretic Analyses of Recombinant Human SHBG

Since CHO cells produced relatively more Con A^- SHBG than BW-1 cells, 10 ml of confluent CHO cell culture medium was subjected to Con A chromatography to separate sufficient amounts of both forms of SHBG. Fractions containing either Con A^+ or Con A^- forms of recombinant SHBG were pooled separately and

Figure 3.5 Con A chromatography of human SHBG in culture medium and serum. Culture medium was collected from CHO (A) and BW-1 (B) cells transfected with a human SHBG cDNA expression construct, and from untransfected wild-type cells as controls. Samples (1 mL) of culture medium and serum (diluted 1:200) were applied under gravity to 5 mL Con A-agarose columns and 1 mL fractions were collected. The *arrows* designate the first fraction where bound protein was eluted with 250 mM mannopyranoside. Aliquots (100 μL) of each fraction were measured for human SHBG by an IRMA.


A
175
150
125
100
75
50
25
0
fmoles SHBG/ mL
Transfected CHO
Serum
Wild-type CHO
0
5
10
15
20
25
Fractions (mL)


B
350
300
250
200
150
100
50
0
fmoles SHBG/ mL
Transfected BW-1
Serum
Wild-type BW-1
0
5
10
15
20
25
Fractions (mL)

concentrated to 2 pmol SHBG/mL for analyses by PAGE and western blotting. Under denaturing conditions, Con A^+ SHBG, purified human SHBG, and SHBG in human serum resolved identically into two subunits with apparent molecular weights of 51,000 and 47,000 (Figure 3.6A). The Con A^- SHBG resolved into two slightly larger subunits with apparent molecular weights of 54,000 and 49,000 (Figure 3.6A). Under nondenaturing conditions (Figure 3.6B), the Con A^- SHBG from CHO cell medium migrated as a single protein with a slightly lower relative mobility (R_f=0.22) than Con A^+ SHBG or serum SHBG (R_f=0.24).

3.2.7 Expression of Recombinant Human SHBG in Nude Mice

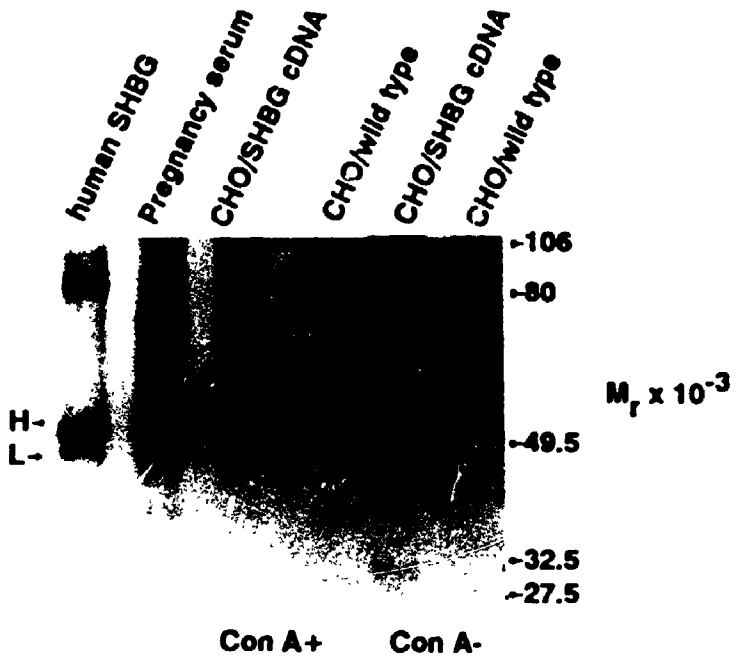
Nude mice have been utilized as hosts for growth studies of human breast cancer cell lines *in vivo* (Shafie and Liotta, 1980; Soule and McGrath, 1980). Therefore, we explored the possibility that CHO cells transfected with an SHBG expression construct could be successfully grown in these mice, and that measurable amounts of SHBG might be secreted into their bloodstream. Ten days after inoculation with SHBG-expressing CHO cells (see 2.15), a highly vascularized mass of cells had grown at the site of injection and blood was withdrawn from the mice. Mouse serum contained 35 pmol human SHBG/mL as measured by an IRMA which is specific for human SHBG, and this clearly indicated that the CHO cells could secrete human SHBG into the mouse blood circulation. Interestingly, only 50% of the human SHBG in the mouse blood bound to Con A after column chromatography (Figure 3.7).

3.3 Discussion

Two expression constructs were generated and used to produce human SHBG in mammalian cell lines, and the ability of these cells to secrete and glycosylate the recombinant protein was compared. Clearly, the pRc/CMV eukaryotic expression vector was much more effective at producing recombinant SHBG in transfected cell lines for several reasons. Unlike the metallothionein I promoter in pBPV which is activated only in the presence of toxic heavy metals, the CMV promoter is constitutively active. Furthermore, pBPV has no selectable marker and therefore

Figure 3.6 Western blots of natural and recombinant human SHBG. Culture medium from CHO cells expressing human SHBG was initially passed through a Blue-Sepharose column to remove albumin and then fractionated by Con A chromatography. Samples of Con A-fractionated human SHBG from CHO cells that were subjected to denaturing (A) and nondenaturing (B) PAGE (7.5% resolving gels), are indicated *below* each blot. The blots were incubated with a primary rabbit polyclonal antiserum against human SHBG and subsequently with a secondary goat anti-rabbit IgG antibody linked to alkaline phosphatase. All other western blots in this thesis were developed using the same antibodies. Culture medium from untransfected wild-type CHO cells was pretreated and analyzed in the same way, as a control. Purified human SHBG and human pregnancy serum were included as positive controls. In (A), positions of the size markers are listed on the *right* and positions of heavy (*H*) and light (*L*) subunits are shown on the *left*.

A


human SHBG
Pregnancy serum
CHO/SHBG cDNA
CHO/wild type
CHO/SHBG cDNA
CHO/wild type
~106
~80
~49.5
~32.5
~27.5
H-
L-
M_r x 10^{-3}
Con A+
Con A-

B

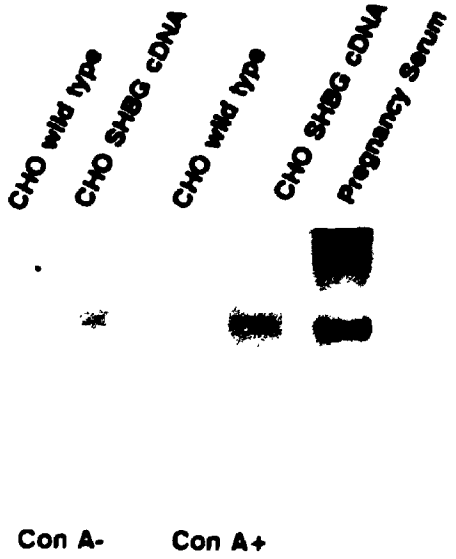
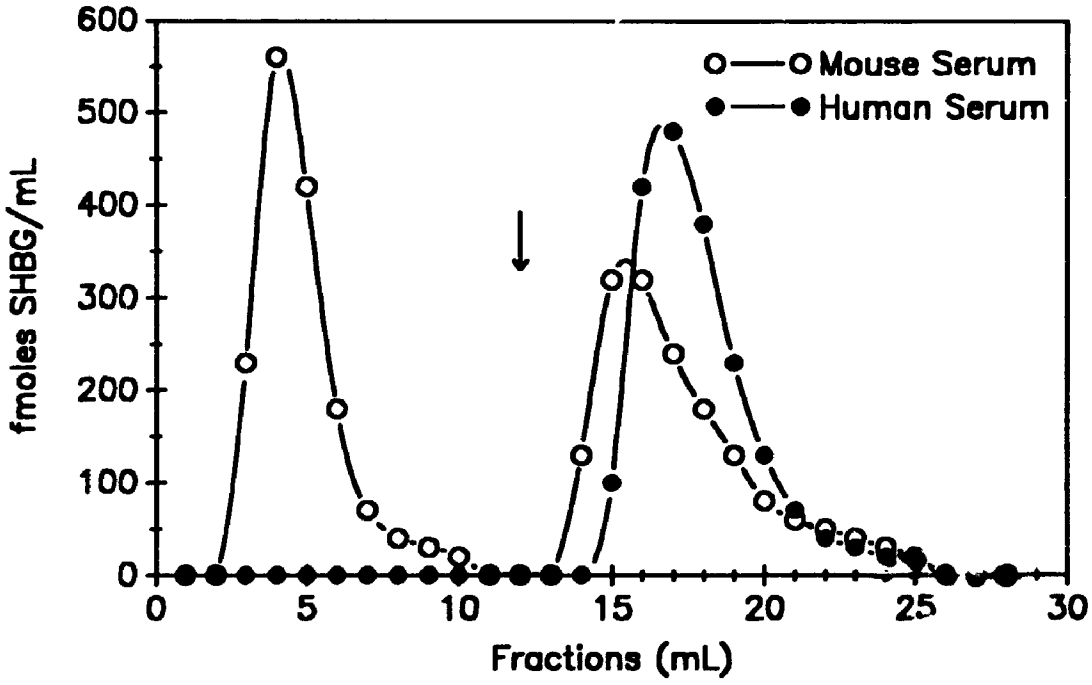

CHO wild type
CHO SHBG cDNA
CHO wild type
CHO SHBG cDNA
Pregnancy Serum
Con A-
Con A+

Figure 3.7 Con A chromatography of SHBG in mouse and human serum. Blood was withdrawn from an adult male nude mouse injected with SHBG-producing CHO cells 10 days earlier, and serum was isolated. Samples (1 mL) of mouse serum (diluted 1:10) and human serum (diluted 1:200) were applied under gravity to 5 mL Con A-agarose columns and 1 mL fractions were collected. The *arrow* designates the first fraction where bound protein was eluted with 250 mM mannopyranoside. Aliquots (100 μL) of each fraction were measured for human SHBG by an IRMA.


600
500
400
300
200
100
0
fmoles SHBG/mL
0
5
10
15
20
25
30
Fractions (mL)
Mouse Serum
Human Serum

had to be co-transfected with the pSV2neo vector, which probably resulted in the growth of many neomycin-resistant colonies lacking the SHBG expression construct.

The recombinant SHBG transcripts in the CHO and BW-1 cells are larger than human hepatic SHBG or testicular ABP mRNAs, which have been estimated to be about 1.6 kb in length (Hammond *et al.*, 1989), and there are two possible explanations for this. First, the start of transcription from the CMV promoter may result in a longer 5' untranslated region than normally associated with the natural SHBG mRNAs. Second, the hepatic SHBG and testicular ABP mRNAs are characterized by short 3' untranslated sequences (Hammond *et al.*, 1989; Hammond *et al.*, 1987), while the recombinant transcript may incorporate the bovine growth hormone polyadenylation signal encoded by the expression vector. Although the relative amount of SHBG mRNA appears to be higher in CHO cells, the BW-1 cells secrete about twice as much SHBG into the culture medium. Therefore, if the amounts of ribosomal RNA in these two cell types are equivalent, it would appear that the BW-1 hepatoma cells express the human SHBG cDNA more efficiently and this was not entirely unexpected since SHBG is actively synthesized by hepatocytes *in vivo*.

Both cell types secreted SHBG into the medium up to concentrations of 4 nM, which were sufficient for detailed physicochemical analyses of the protein. In contrast, another human SHBG expression system has been developed by others (Hagen *et al.*, 1992) using the baby hamster kidney (BHK) cell line, but the recombinant product was so poorly secreted from these cells that it had to be partially-purified from 1.5 L of culture medium for analysis. Rat ABP has also been transiently expressed using a monkey kidney cell (COS) cell line, but again the level of rat ABP secreted by these cells was 100-fold lower than our system and the culture medium had to be concentrated 30-fold for analyses (Joseph *et al.*, 1992). Given the problems associated with the presence of bovine SHBG in FBS, our expression system is undoubtedly the most efficient described so far for the study of recombinant extracellular sex steroid-binding proteins.

Like SHBG in human serum, recombinant human SHBG in the culture medium exhibits a steroid-binding capacity that closely resembled its concentration determined immunochemically. A close correlation between these independent measurements suggests that the recombinant product has undergone appropriate folding and dimerization during synthesis to create a functional steroid-binding site. Furthermore, the fact that recombinant SHBG is indistinguishable from serum SHBG in the assay for homodimer formation suggests the conformation-dependent epitope on each recombinant subunit is intact (Hammond *et al.*, 1985).

When subjected to Con A chromatography, almost all the SHBG in human serum binds to the column matrix (Hsu and Troen, 1978). By contrast, only about half of the ABP in human testicular homogenates binds to this lectin under similar conditions (Hsu and Troen, 1978; Cheng *et al.*, 1985b). These earlier observations suggested that tissue-specific differences in SHBG glycosylation may occur *in vivo*. The results of our expression studies clearly support this because transfected BW-1 hepatoma cells produce a higher percentage of a Con A^+ form of SHBG than transfected CHO cells. It is interesting that the BW-1 cells secrete a Con A^- form and it remains possible that human hepatocytes also produce this form of SHBG *in vivo*. Although, a human hepatocarcinoma (HepG2) cell line secretes SHBG with the same Con A-binding properties as plasma SHBG (Khan *et al.*, 1981), it remains to be determined whether this transformed cell line accurately reflects the activity of normal cells *in vivo*. If a Con A^- form is secreted into the blood, it must be quickly removed from the circulation because human SHBG in serum interacts quantitatively with Con A. The significance of these glycosylation differences is not known, but specific carbohydrate chains may be physiologically important. For instance they may interact with an SHBG receptor on the plasma membranes of some cell types (Strel'chyonok *et al.*, 1984b; Hryb *et al.*, 1985; Porto *et al.*, 1992b).

The Con A profile of human SHBG secreted by CHO cells into nude mouse blood is different than human SHBG secreted into the culture medium and this could be due to the growth of the cells in the presence of endogenous hormones

and growth factors, which could affect the glycosylation of SHBG. Although, the glycosylation status of human SHBG in the blood remains unchanged during pregnancy, some hepatocyte-derived plasma glycoproteins contain more branched, sialylated oligosaccharides during pregnancy (Rademacher *et al.*, 1988). As an example, a human CBG variant, which contains only *N*-linked triantennary oligosaccharides and does not bind to Con A, is present in the blood during pregnancy (Strel'chyonok *et al.*, 1984a). Thus, it is possible that hepatocytes secrete SHBG with more branched oligosaccharides, but these are not detected in serum samples because they are efficiently removed from the blood. Therefore, the mouse study is interesting since SHBG containing more branched carbohydrate structures produced by CHO cells can accumulate in the bloodstream. However, it could be argued that mice do not normally contain a circulating SHBG, and its tissues may not remove SHBG from the blood in the same way as human tissue.

In conclusion, these studies represented the first description of the expression of recombinant human SHBG in mammalian cells, and they demonstrate that the product is essentially immunologically and physicochemically indistinguishable from its natural counterpart. More importantly, a protocol has been devised that results in the constitutive production of recombinant human SHBG, and in conjunction with site-directed mutagenesis, will permit a more detailed molecular analyses of the carbohydrate composition of SHBG, its steroid-binding site, dimerization domain and its interaction with plasma membrane receptors. The demonstration that CHO cells could be successfully grown in nude mice and secrete human SHBG into the bloodstream is important because it provides an *in vivo* model for studies of the clearance of different forms of SHBG from the blood and its possible targeting to different tissues. Furthermore, it might be possible to investigate the effect of circulating human SHBG on the growth of human sex steroid-dependent cell types, which could be grown at a different site in the mouse. Certainly, the generation of SHBG mutants with altered steroid-binding properties or which lack specific carbohydrate chains could be included in these studies.

CHAPTER 4

PHYSICOCHEMICAL ANALYSES OF SHBG GLYCOSYLATION MUTANTS

4.1 Introduction

My initial studies described in the previous chapter showed that human SHBG expressed in different mammalian cell lines is characterized by the same subunit size micro-heterogeneity as SHBG purified from human serum, and that its glycosylation is cell-type specific. It is not known whether these variations in the carbohydrate composition of SHBG are functionally important. As a first step towards addressing this issue, site-directed mutagenesis was employed to selectively remove glycosylation sites from the SHBG molecule and the physicochemical properties of the resultant glycosylation-deficient mutants were examined.

Naturally-occurring human SHBG variants with reduced electrophoretic mobility are frequently found in populations of diverse ethnic origins and are characterized by an additional subunit that is larger than the two subunits normally associated with SHBG in the general population (Van Baelen *et al.,* 1992). The *N*-terminal sequence of these electrophoretic SHBG variants corresponds to that of the normal subunit (Gershagen *et al.,* 1987) and this suggested that an additional carbohydrate chain may account for their increased size (Van Baelen *et al.,* 1992). Therefore, to determine the molecular basis for this variant, genomic DNA and serum SHBG from a mother and three of her daughters, homozygous for this trait, were analyzed.

4.2 Results

4.2.1 Production and Steroid-binding Properties of SHBG Glycosylation-deficient Mutants

Site-directed mutagenesis was used to introduce nucleotide changes in a human SHBG cDNA (Figure 2.1, construct #6) which encode for substitutions of amino acid that are normally sites for glycosylation (Table 2.1) and the mutated SHBG cDNAs were transfected into CHO cells for expression of the mutant proteins. Culture medium was routinely collected from cells grown to confluence and SHBG levels measured by a steroid-binding capacity assay and IRMA revealed that removal of any one of the three carbohydrate chains normally associated with SHBG does not influence its production and/or secretion (Table 4.1). However, the

Table 4.1 Measurements of the concentration and steroid-binding properties of wild-type SHBG and SHBG glycosylation-deficient mutants. The concentration of SHBG in the culture medium were assessed using an immunoradiometric assay (IRMA) specific for human SHBG and a steroid-binding capacity assay (BCA). Dissociation constants (k_d) were measured by Scatchard analysis and the relative affinity of SHBG for each steroid was defined as the relative amount of competitor required to displace 50% of the labeled ligand when compared to DHT. All steroid-binding analyses were performed at 0°C and used [^{3}H]DHT as labeled ligand and DCC to separate bound and free steroid. Abbreviations are: T7A, $Thr^7 \rightarrow Ala$; N351Q, $Asn^{351} \rightarrow Gln$; N367Q, $Asn^{367} \rightarrow Gln$.

PRODUCT	SHBG LEVEL (pmol/mL)		AFFINITY FOR DHT	RELATIVE BINDING AFFINITY		
	IRMA	BCA	k_d (nM)	DHT	T	E_2
Wild-type	4.8	5.3	0.36	100	19	6
T7A	4.6	5.2	0.33	100	20	7
N351Q	4.6	5.2	0.27	100	16	7
N367Q	4.4	4.7	0.28	100	20	7
N351Q + N367Q	1.2	1.6	0.33	100	19	8
T7A + N351Q + N367Q	1.0	1.3	0.35	100	19	7

elimination of both *N*-linked oligosaccharides clearly reduces the levels of SHBG secreted into the culture medium, irrespective of the presence of an *O*-linked oligosaccharide (Table 4.1), and similar results were obtained in two separate transfection experiments.

A strong correlation (r = 0.99) between the concentrations of SHBG mutants in the culture medium was obtained by IRMA and steroid-binding capacity measurements (Table 4.1), suggesting that the mutant proteins are capable of interacting appropriately with steroid. Scatchard analyses (Scatchard, 1949) and measurements of their relative steroid-binding affinities confirmed that the glycosylation-deficient mutants bound steroid ligands with comparable affinity and specificity as wild-type SHBG (Table 4.1).

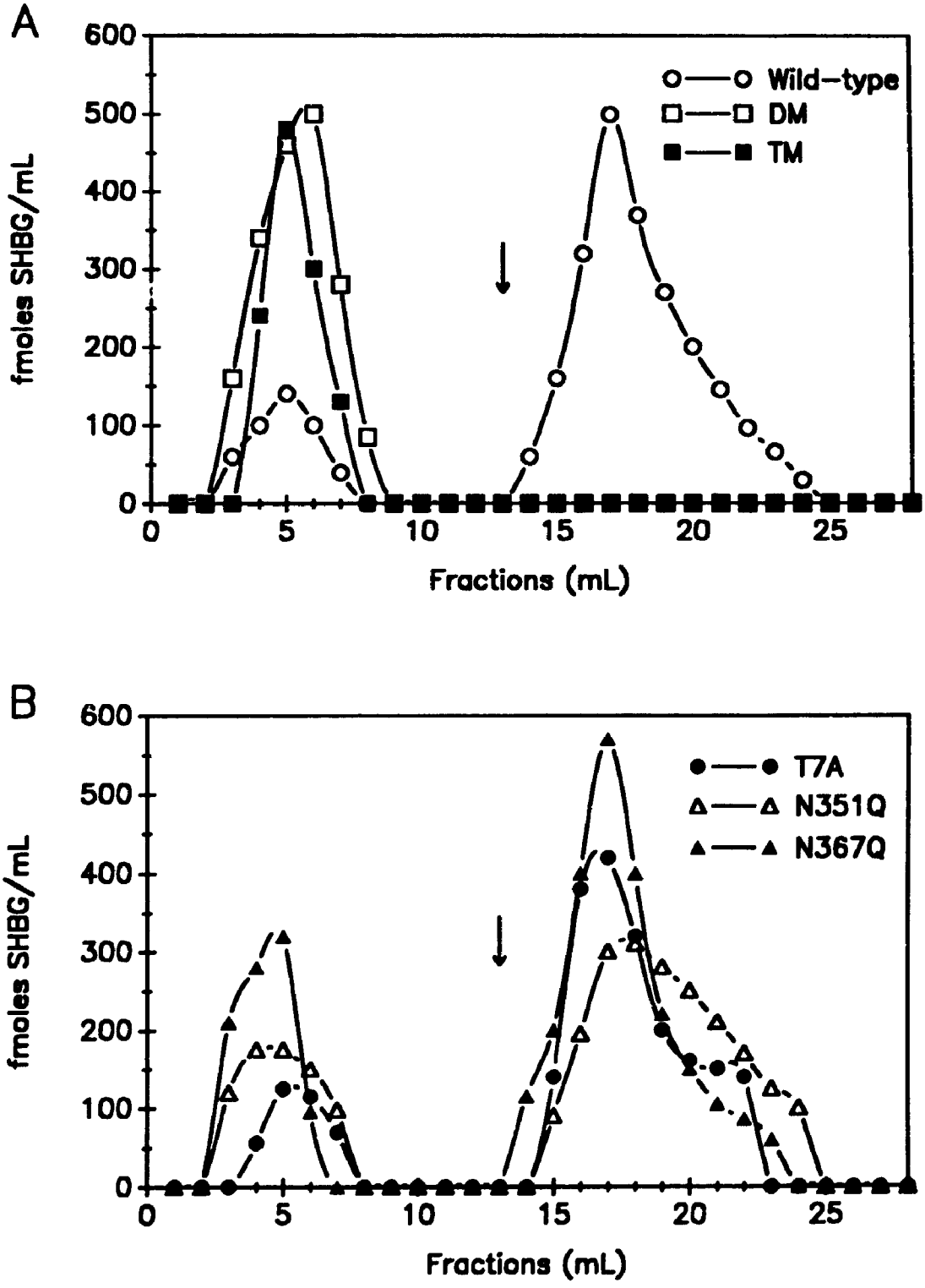
4.2.2 Concanavalin A-binding Properties of SHBG Glycosylation-deficient Mutants

Removal of both *N*-linked carbohydrate chains, in the presence or absence of the *O*-linked oligosaccharide at position 7, resulted in a total loss of Con A-binding activity, when compared to wild-type SHBG (Figure 4.1A). The SHBG mutant lacking only the *O*-linked oligosaccharide chain at position 7 (T7A) exhibited a similar Con A chromatographic profile to wild-type SHBG produced by CHO cells with approximately 15% failing to interact with Con A (Figure 4.1A, 1B). By contrast, removal of *N*-linked oligosaccharide chains individually (N351Q, N367Q) increased the proportion of SHBG that did not interact with the column to 26% for N351Q and 31% for N367Q (Figure 4.1B).

4.2.3 Electrophoretic Properties of SHBG Glycosylation-deficient Mutants

Human SHBG produced by CHO cells, which does not bind to Con A (Con A$^-$), is almost exclusively composed of subunits that migrate with slightly lower mobility than those of SHBG that bind to Con A (Figure 3.6A). As a result, when unfractionated culture medium containing recombinant SHBG is examined by denaturing-PAGE and western blotting it is difficult to determine subunit sizes. Therefore, to optimize the resolution between subunits associated with the

Figure 4.1 Con A chromatography of wild-type SHBG and SHBG mutants lacking either multiple (A) or individual (B) carbohydrate chains. (A) *DM*, double mutant (N351Q + N367Q); *TM*, triple mutant (T7A + N351Q + N367Q). Samples (1.5 mL) of culture medium were applied under gravity to 5 mL Con A-agarose columns and 1 mL fractions were collected. The *arrows* designate the first fraction where bound protein was eluted with 250 mM mannopyranoside. Aliquots (100 μL) of each fraction were measured for human SHBG by an IRMA.

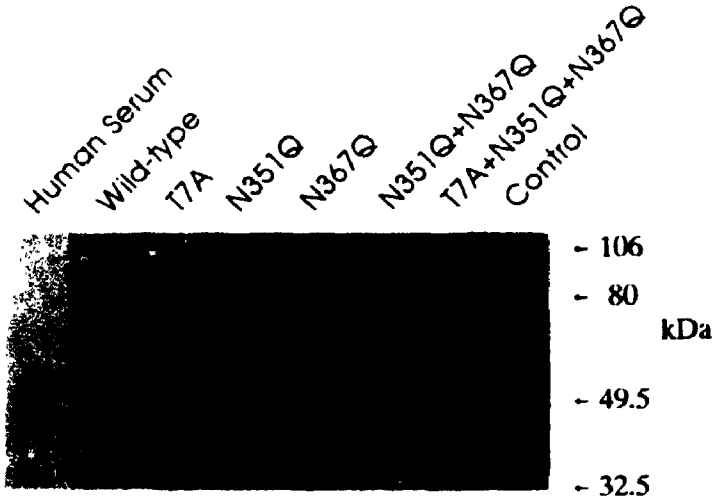

A
600
500
400
300
200
100
0
fmoles SHBG/mL
Wild-type
DM
TM
0
5
10
15
20
25
Fractions (mL)
B
600
500
400
300
200
100
0
fmoles SHBG/mL
T7A
N351Q
N367Q
0
5
10
15
20
25
Fractions (mL)

predominant form of SHBG produced by CHO cells, and which most closely resembles SHBG in serum samples, the Con A-binding (Con A^+) forms of those proteins containing one or more *N*-linked oligosaccharide chain were compared separately from the Con A^- forms. In addition, an immunopurification step (see 2.13) was used to concentrate the SHBG in the samples prior to analysis by western blotting. This demonstrated that removal of either *N*-linked carbohydrate chain (N351Q, N367Q) eliminated the size heterogeneity associated with wild-type SHBG, and resulted in a single subunit that migrates with a similar mobility as the light subunit normally present as a minor component of wild-type SHBG produced by CHO cells, or SHBG in serum samples (Figure 4.2A). Mutation of both consensus sites for *N*-glycosylation further reduced the molecular size of this single subunit. By contrast, the substitution of Thr^7 with an alanine (T7A) resulted in a reduction in the molecular size of both heavy and light subunits associated with wild-type SHBG with no apparent change in the relative amounts of the two subunit forms (Figure 4.2A). A further reduction in the size of the subunit associated with the mutant lacking both *N*-linked oligosaccharides was observed when its codon for Thr^7 was also mutated to prevent *O*-linked glycosylation (Figure 4.2A).

The Con A^- forms of wild-type SHBG and the T7A, N351Q and N367Q SHBG mutants were also analyzed by denaturing-PAGE to determine if their subunits differed in size when compared to the corresponding subunits that bound to Con A (Figure 4.2B). As expected, the heavy and light subunits comprising the Con A^+ forms of wild-type and T7A SHBG were clearly smaller than their corresponding Con A^- counterparts. In addition, the Con A^+ forms of mutants with only one *N*-linked oligosaccharide (N351Q, N367Q) migrated as one isoform while the Con A^- forms resolved into two electrophoretic species. For illustrative purposes, the Con A^+ and Con A^- forms of the N367Q mutant are compared directly in Figure 4.3. As expected, the Con A^- form of this mutant is composed predominantly of a subunit (49 kDa) that is larger than the corresponding subunit associated with the Con A^+ form (47 kDa), which must contain a biantennary chain. In addition, a less

Figure 4.2 Western blots of human serum SHBG, wild-type SHBG and the SHBG glycosylation-deficient mutants. Wild-type SHBG and SHBG mutants lacking a single oligosaccharide chain were fractionated by Con A chromatography. All samples were immunopurified (see 2.13), prior to denaturing-PAGE (7.5% resolving gel). (A) The subunit sizes of SHBG mutants lacking multiple carbohydrate chains and the Con A^+ fractions of serum, wild-type, T7A, N351Q and N367Q SHBG are indicated *below* the blot (*Subunits*). The size of the nondenatured proteins were determined by gel filtration on a Superose 6-FPLC column and are indicated *below* the blot (*Dimer*). Conditioned medium from untransfected CHO cells was analyzed as a control. (B) The Con A^+ forms of wild-type, T7A, N351Q and N367Q SHBG were compared to their counterparts, and to SHBG mutants lacking multiple carbohydrate chains, that did not bind Con A (Con A^-), as indicated *below* the blot. Positions of the size markers are listed to the *right* of both blots.

A



Subunits (kDa)	50 47	51 47	49 45	47	47	43	41	
Dimer (kDa)	98	98	98	84	98	80	77	

B

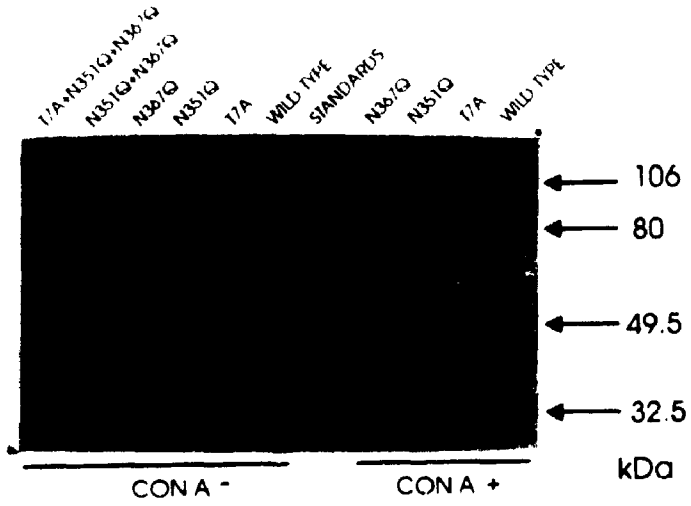
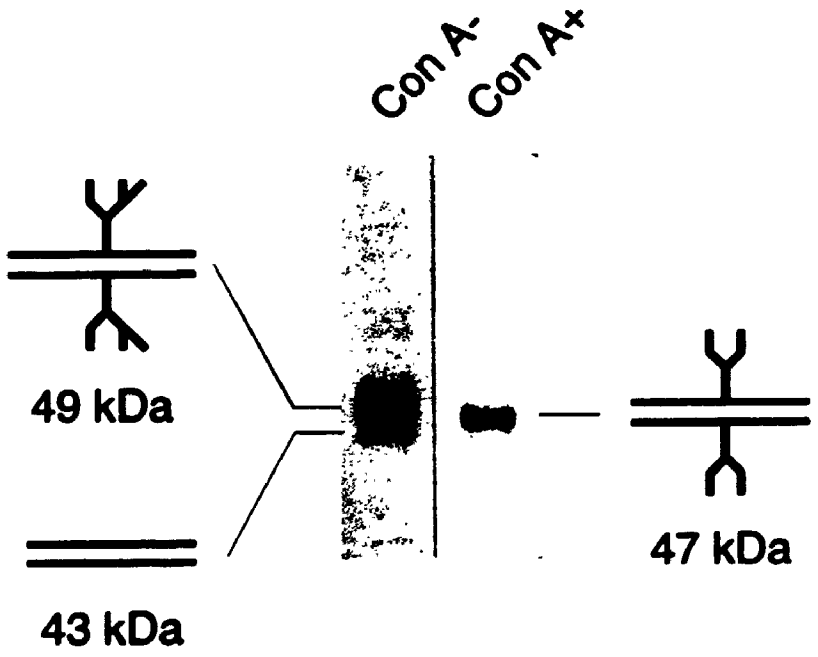


Figure 4.3 Western blot of an SHBG mutant containing one *N*-glycosylation site. The N367Q SHBG mutant was fractionated by Con A chromatography and the samples were immunopurified prior to analysis by denaturing-PAGE (7.5% resolving gel). The size and the *N*-glycosylation content (***branched lines***) of each subunit isoform (***straight lines***) comprising SHBG dimers are indicated.


Con A-
Con A+
49 kDa
43 kDa
47 kDa

abundant 43 kDa subunit is also present in the Con A^- form, which is smaller than the corresponding Con A^+ subunit (Figure 4.3), and is equal in size to a subunit lacking both *N*-linked oligosaccharides (Figure 4.2).

4.2.4 Size Exclusion Chromatography

The human SHBG glycosylation-deficient mutants produced by transfected CHO cells displayed differences in retention during gel filtration, and their sizes were calculated by comparison with elution volumes of molecular size standards (Figure 4.2A). As expected, either the disruption of both *N*-glycosylation sites (M_r=80,000) or in combination with the substitution of alanine for threonine at position 7 (M_r=77,000) resulted in substantial reductions in molecular size, when compared to wild-type SHBG (M_r=98,000). Interestingly, the N351Q and N367Q mutants differed with respect to their elution volumes and their calculated molecular sizes were 84,000 and 98,000, respectively (Figure 4.2A).

4.2.5 Genetic Analyses of an SHBG Electrophoretic Variant

In a collaborative study to determine the molecular basis of an SHBG electrophoretic variant, genomic DNA samples from an individual suspected to be homozygous for this trait and three of her offspring were subjected to PCR amplification of their SHBG coding regions by Dr. Stephen Power. Sequence analysis of genomic DNA (Hammond *et al.*, 1987) revealed a point mutation (GAC→AAC) in the codon for residue 327 in the SHBG polypeptide sequence, which results in the substitution of Asp(D)→Asn(N), generating an additional consensus site for *N*-glycosylation at this position. In total, 12 PCR products of exon 8 (encoding residues 325-373) from these four individuals were examined and the same mutation was identified. To confirm that the additional consensus site for *N*-glycosylation is utilized, I was responsible for introducing this mutation into a human SHBG cDNA by site-directed mutagenesis, and to study the physicochemical properties of this SHBG mutant (D327N) expressed in CHO cells.

4.2.6 Physicochemical Properties of an SHBG Electrophoretic Variant

Medium collected from CHO cells expressing either the wild-type SHBG or the D327N SHBG variant was analyzed by an IRMA and a steroid-binding capacity assay to determine their concentration. The measurements obtained using both assays were equivalent and this suggested that D327N SHBG bound steroids appropriately. This was confirmed by Scatchard analysis which demonstrated that both wild-type and D327N SHBG have essentially identical affinities for [^{3}H]DHT (Figure 4.4), with dissociation constants of 0.52 nM and 0.57 nM, respectively.

4.2.7 Concanavalin A-binding Properties of an SHBG Electrophoretic Variant

Culture medium from transfected CHO cells was analyzed by Con A chromatography and the elution profiles of wild-type SHBG and the D327N mutant were compared to that obtained for SHBG in diluted serum samples containing the natural electrophoretic variant (Figure 4.5). As observed for normal SHBG in human serum (Figure 3.5, 3.8), the variant SHBG in the serum samples bound quantitatively to the Con A column, while proportionally less of wild-type (85%) and D327N (54%) SHBG produced by CHO cells bound to this lectin (Figure 4.5).

4.2.8 Electrophoretic Properties of an SHBG Electrophoretic Variant

When subjected to denaturing-PAGE and western blot analysis, normal SHBG in serum resolved into heavy and light subunits of 52 and 48 kDa, respectively (Figure 4.6A). These subunits could also be detected in serum containing the electrophoretic variant, but the majority of the immunoreactive SHBG in this sample appeared as a super-heavy subunit of 56 kDa. The Con A$^+$ forms of wild-type and D327N SHBG produced by CHO cells both exhibit heavy and light subunits of similar size to those associated with normal SHBG in serum, but an extra immunoreactive subunit of approximately 60 kDa was also detected in the Con A$^+$ fraction of the D327N SHBG mutant (Figure 4.6A). Furthermore, as previously noted for wild-type SHBG, the subunits of the Con A$^+$ forms of D327N were smaller than their counterparts that did not bind to Con A (Figure 4.6B). The Con A$^-$ forms of wild-type SHBG also comprised a third electrophoretic species that is

Figure 4.4 Scatchard plots of wild-type and variant (D327N) SHBG produced by CHO cells. The dissociation constants (k_d) were measured at 0°C using [^{3}H]DHT as the labeled ligand and DCC to separate bound and free steroid. Each *data point* represents the mean value of triplicate measurements. Wild-type SHBG, k_d=0.52 nM; D327N SHBG, k_d=0.57 nM.

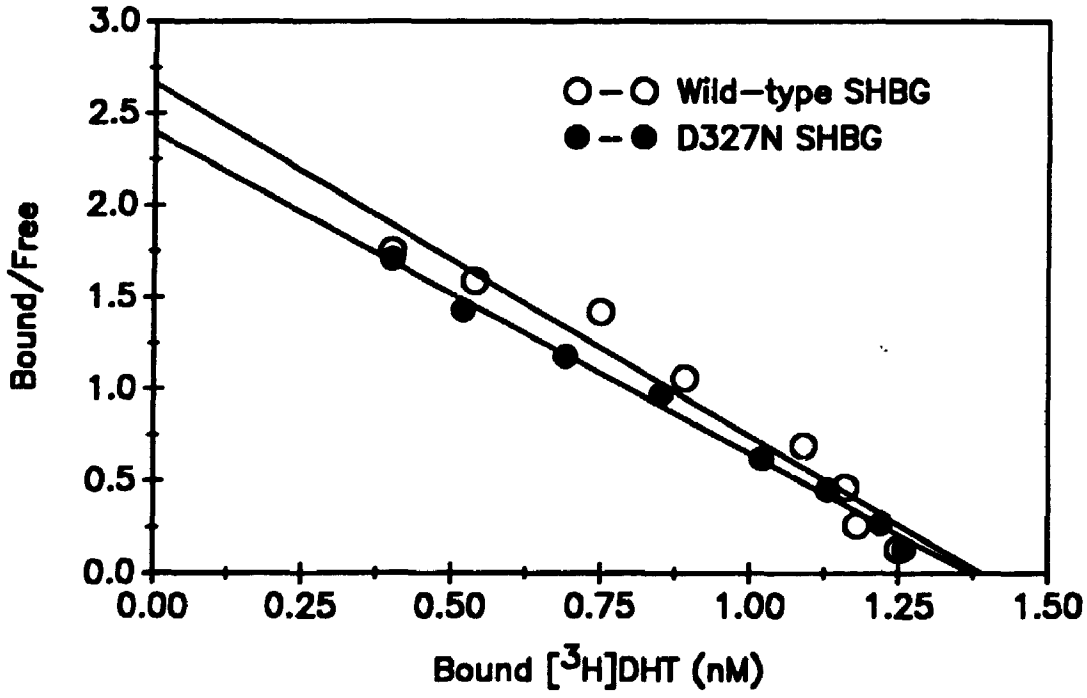

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Bound/Free
O–O Wild–type SHBG
●–● D327N SHBG
0.00
0.25
0.50
0.75
1.00
1.25
1.50
Bound [3H]DHT (nM)

Figure 4.5 Con A chromatography of wild-type and D327 SHBG produced by CHO cells, and variant SHBG in human serum. Samples (1 mL) of culture medium or serum (diluted 1:10) were applied under gravity to 5 mL Con A-agarose columns and 1 mL fractions were collected. The *arrow* designates the first fraction where bound protein was eluted with 250 mM mannopyranoside. Aliquots (100 μL) of each fraction were measured for human SHBG by an IRMA.

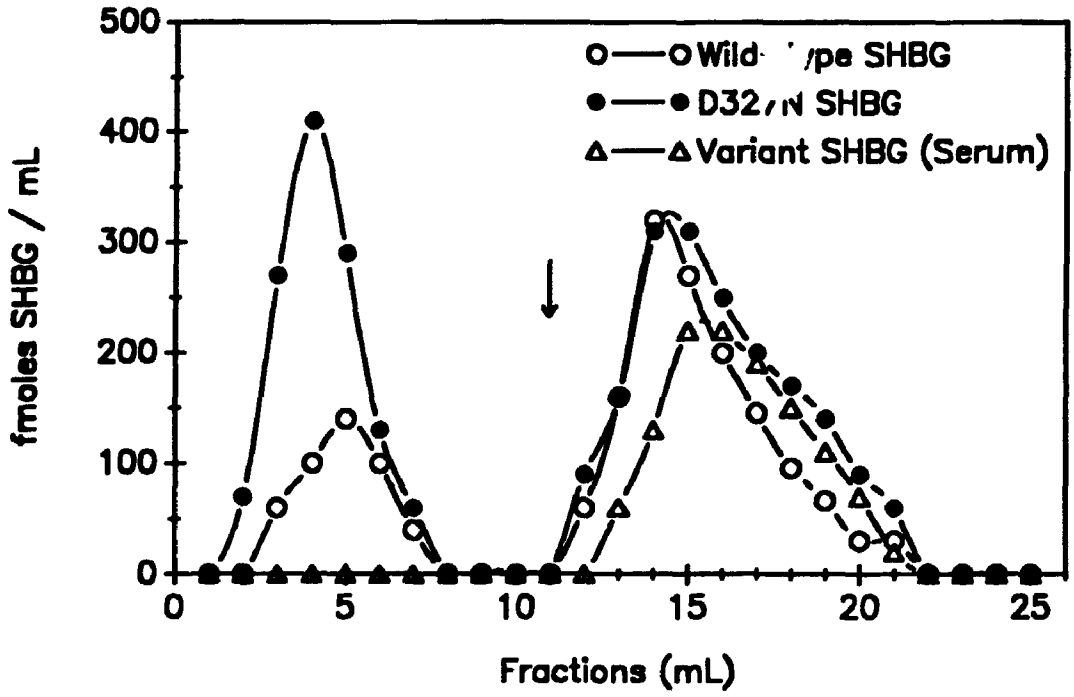
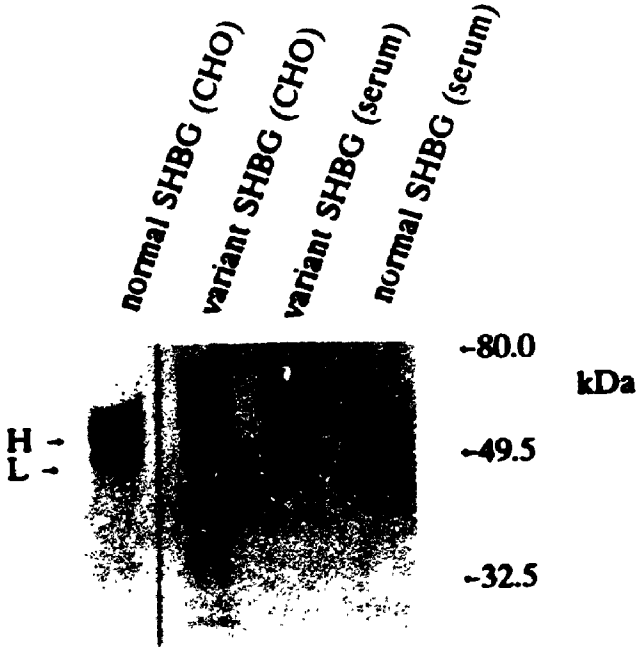
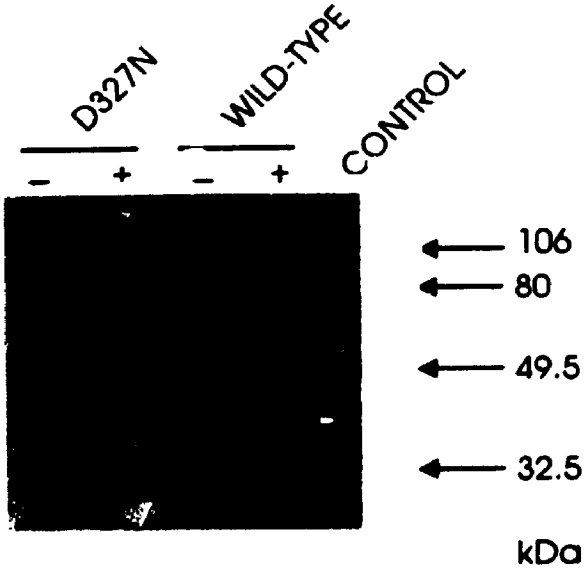

500
400
300
200
100
0
fmoles SHBG / mL
0
5
10
15
20
25
Fractions (mL)
Wild-type SHBG
D32N SHBG
Variant SHBG (Serum)

Figure 4.6 Western blots of wild-type and D327N SHBG in CHO cell culture medium, and normal and variant SHBG in human serum. The recombinant proteins were first fractionated by Con A chromatography and then all samples were immunopurified prior to analysis by denaturing-PAGE (7.5% resolving gel). (A) The Con A^+ forms of the recombinant SHBG samples were directly compared with the human serum samples. The positions of the heavy (*H*) and light (*L*) subunits associated with normal SHBG are indicated to the *left*. (B) The Con A^+ forms (+) of wild-type and D327N SHBG were compared to their counterparts which did not bind to Con A (-) and conditioned medium from untransfected CHO cells was used as a control. Positions of the size markers are listed to the *right* of both blots.

A


normal SHBG (CHO)
variant SHBG (CHO)
variant SHBG (serum)
normal SHBG (serum)
~80.0
kDa
~49.5
~32.5
H
L

B


D327N
WILD-TYPE
CONTROL
–
+
–
+
106
80
49.5
32.5
kDa

smalier in size than both the normal heavy and light subunits, but this species is not detectable in the Con A$^-$ fraction of the D327N mutant isolated from culture medium (Figure 4.6B).

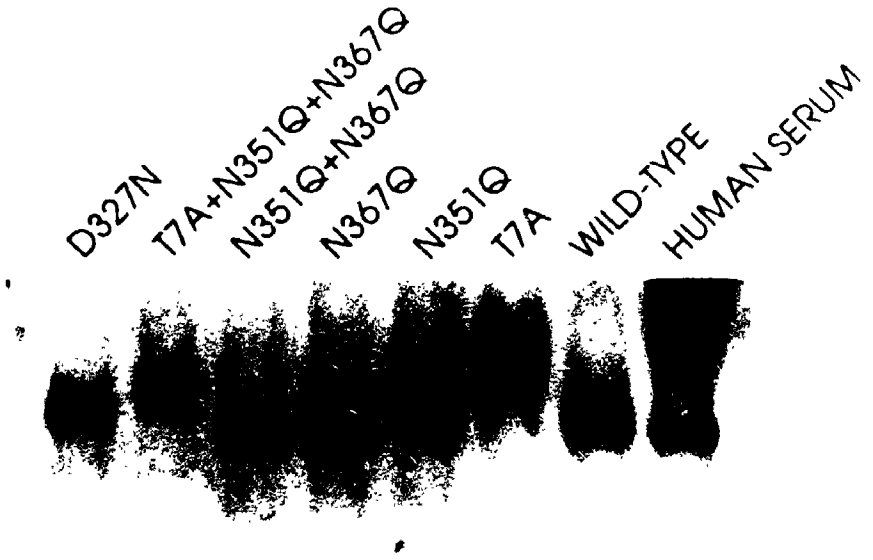
4.2.9 Nondenaturing Electrophoretic Properties of SHBG Glycosylation Mutants

The relative electrophoretic mobilities of the glycosylation mutants were compared to wild-type recombinant SHBG and serum SHBG. This was done because glycosylation status can influence the size, conformation and charge of SHBG which might be reflected by differences in electrophoretic mobility. Clearly, the two mutants lacking an *O*-linked oligosaccharide at position 7 resulted in proteins with the least mobility (Figure 4.7). Furthermore, the mutant completely lacking carbohydrates exhibited a lower mobility than the SHBG mutant with the greatest carbohydrate content (D327N). The N351Q and N367Q SHBG mutants had different mobilities despite the loss of a single *N*-linked oligosaccharide in both cases (Figure 4.7).

4.3 Discussion

I have been able to selectively prevent the glycosylation of SHBG at specific sites using site-directed mutagenesis of a human SHBG cDNA. For these studies, the various SHBG cDNAs were expressed in a cell line that contains the full complement of enzymes required for both *N*- and *O*-linked glycosylation, and which has been used to express other secreted glycoproteins including human FSH (Galway *et al.*, 1990; Hard *et al.*, 1990) and chorionic gonadotropin (Matzuk *et al.*, 1987; Keene *et al.*, 1989). Although this approach could potentially alter the conformation and function of the recombinant proteins, analyses of the immunological and steroid-binding properties of the SHBG mutants demonstrated that this was probably not the case. The major advantage of using site-directed mutagenesis to prevent glycosylation is that it overcomes the problems associated with the use of chemical or enzymatic deglycosylation techniques; these may include incomplete or non-specific modifications of carbohydrate chains, and an inability to study the potential role of carbohydrates in the folding process during synthesis

Figure 4.7 Western blot of wild-type SHBG, the SHBG glycosylation mutants and human serum SHBG. Culture medium samples containing the recombinant proteins, and human SHBG in serum (diluted 1:200) were subjected to nondenaturing-PAGE (7.5% resolving gel), prior to western blotting.

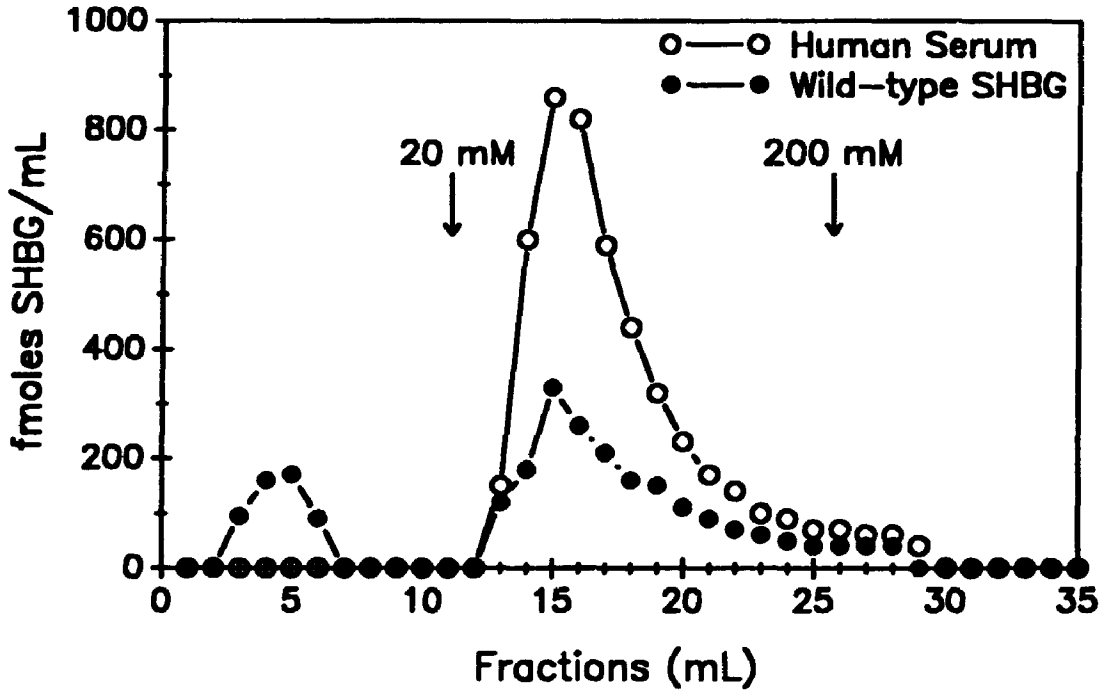

D327N
T7A+N351Q+N367Q
N351Q+N367Q
N367Q
N351Q
T7A
WILD-TYPE
HUMAN SERUM

(Petra *et al.*, 1992).

The lack of individual carbohydrate chains had no influence on the production of SHBG by CHO cells. However, the elimination of both *N*-linked oligosaccharides reduced the levels of SHBG secreted into the culture medium, irrespective of the presence or absence of the *O*-linked carbohydrate chain. Similar results have been obtained as a result of the expression of rat ABP mutants lacking *N*-glycosylation sites in COS cells (Joseph *et al.*, 1992). Therefore, glycosylation does not appear to be an absolute requirement for the biosynthesis or secretion of these proteins, but may affect the efficiency of these processes. These data also demonstrate that the lack of specific carbohydrate chains, or an additional carbohydrate chain at position 327 have no effect on the steroid-binding properties of SHBG. Furthermore, all of the mutants reacted appropriately with both the polyclonal and monoclonal antibodies against human SHBG, and this suggests that the lack of specific carbohydrate chains, the presence of an extra oligosaccharide chain at residue 327, or the amino-acid substitutions we have introduced do not adversely affect the overall conformation of the proteins.

Lectin-affinity chromatography has been used to define the types of carbohydrate moieties attached to sex-steroid binding proteins (Danzo and Black, 1990a; Danzo and Black, 1990b). Glycoproteins require only one *N*-linked biantennary or a high mannose-type oligosaccharide chain to bind to Con A, and those containing high mannose-type chains require substantially greater concentrations of mannopyranoside (>100 mM) to be displaced from this lectin (Narasimhan *et al.*, 1979; Krusius *et al.*, 1976). Since the Con A-binding form of SHBG produced by CHO cells could be completely eluted from the Con A column with 20 mM mannopyranoside within three column volumes, in the same way as serum SHBG (Figure 4.8), it is unlikely that there are any high mannose-type chains associated with these proteins. It should also be noted that when CHO cells expressing wild-type SHBG were grown in 10% FBS, 74% was shown to bind Con A (Figure 3.5). However, when these cells were grown in DMEM alone for 3 days,

Figure 4.8 Con A chromatography of recombinant human SHBG and SHBG in human serum. Samples (1 mL) of culture medium or serum (diluted 1:200) were applied under gravity to 5 mL Con A-agarose columns and 1 mL fractions were collected. The *arrows* designate the first fraction where bound protein was eluted with 20 mM or 200 mM mannopyranoside as indicated. Aliquots (100 μL) of each fraction were measured for human SHBG by an IRMA.


1000
800
600
400
200
0
fmoles SHBG/mL
0
5
10
15
20
25
30
35
Fractions (mL)
Human Serum
Wild-type SHBG
20 mM
200 mM

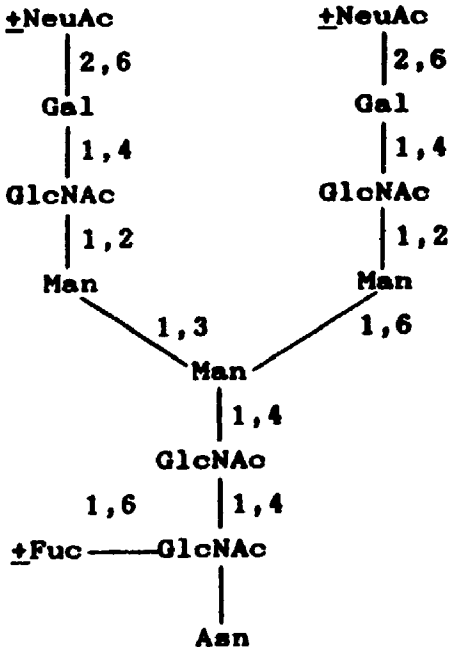
the percentage of wild-type SHBG that bound to Con A increased to 85%, indicating that the constituents of FBS can influence the post-translational processing of SHBG.

It is interesting that mutants lacking a single *N*-linked carbohydrate chain comprise proportionally greater amounts of SHBG which do not interact with Con A (26-31%), when compared to wild-type SHBG (15%) or SHBG lacking only the *O*-linked oligosaccharide (15%). These data show that in CHO cells, SHBG mutants containing a single *N*-glycosylation site on both subunits will receive a triantennary carbohydrate chain (Figure 4.9) 30% of the time. Moreover, when two *N*-glycosylation consensus sites are present per subunit, 15% of wild-type SHBG does not interact with Con A, and this is similar to the expected value of 9% (30% x 30%). However, when D327N SHBG containing three *N*-glycosylation consensus sites per subunit was expressed in CHO cells, it was anticipated that only 2.7% (30% x 30% x 30%) of this mutant would not bind to Con A, but in fact 46% of D327N SHBG did not interact with this lectin. Although, there is no doubt that glycosylation is cell-type specific, it is likely that the number and positioning of *N*-glycosylation sites within SHBG can influence the processing of carbohydrate chains.

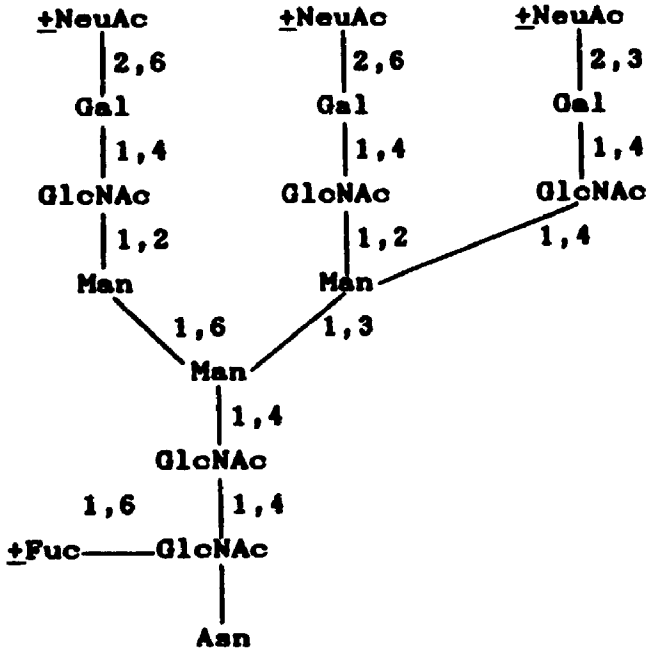
When human SHBG in serum is examined by denaturing-PAGE, the subunits resolve into heavy and light isoforms (Cheng *et al.*, 1983), and the molecular basis for this has been the subject of speculation (Hammond, 1990). The data presented here clearly demonstrate that both consensus sites for *N*-glycosylation on human SHBG can be utilized, and that this subunit size micro-heterogeneity is maintained irrespective of the absence of the *O*-linked carbohydrate at residue 7. Moreover, when either of the *N*-glycosylation sites is mutated, the sizes of the products are essentially identical to the light subunit associated with the natural protein. This suggests that the heavy subunit of SHBG contains two *N*-linked oligosaccharide chains, while only one is present on the light subunit. Furthermore, it is unlikely that the light subunit is a product of limited proteolysis, because the mutant lacking all carbohydrate attachments does not exhibit subunit size heterogeneity, and has

Figure 4.9 Chemical structure of biantennary (A) and triantennary (B) *N*-linked oligosaccharide chains of the *N*-acetyllactosamine type. GlcNAc, *N*-acetylglucosamine; Man, mannose; Gal, galactose; Fuc, fucose; NeuAc, *N*-acetylneuraminic acid. Glycosidic bonds are indicated.

A


±NeuAc
2,6
Gal
1,4
GlcNAc
1,2
Man
1,3
±NeuAc
2,6
Gal
1,4
GlcNAc
1,2
Man
1,6
Man
1,4
GlcNAc
1,6
1,4
±Fuc
GlcNAc
Asn

B


±NeuAc
2,6
Gal
1,4
GlcNAc
1,2
Man
1,6
±NeuAc
2,6
Gal
1,4
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1,2
Man
1,3
±NeuAc
2,3
Gal
1,4
GlcNAc
1,4
Man
1,4
GlcNAc
1,6
1,4
±Fuc
GlcNAc
Asn

a size that corresponds almost exactly to the molecular weight of the SHBG polypeptide (M_r=40,475) based on its amino acid composition.

The advantage of resolving SHBG glycosylation mutants that contain one *N*-glycosylation site by Con A chromatography, prior to analysis by denaturing-PAGE, is that it facilitates interpretation of the carbohydrate content of subunits that constitute particular dimers. For example, when the SHBG mutant containing only one glycosylation site at Asn^{351} (N367Q), is fractionated by Con A chromatography, the Con A^+ form which must contain at least one biantennary carbohydrate chain (Figure 4.9) per dimer, migrates as a single electrophoretic species of 47 kDa, while the form that does not interact with Con A, migrates as two electrophoretic species of 49 kDa and 43 kDa (Figure 4.3). If we assume that the 49 kDa subunit contains a more processed (triantennary) oligosaccharide (Figure 4.9), and the 43 kDa subunit contains no *N*-linked carbohydrates chains, it must therefore follow that both subunits that comprise SHBG dimers in the Con A-bound fraction must each contain only one biantennary oligosaccharide (Figure 4.3), because the other two electrophoretic isoforms are not detected. This implies that after subunit association, which probably occurs in the endoplasmic reticulum, both SHBG subunits of a given dimer are glycosylated and processed in exactly the same way.

Analysis of the coding sequence for human SHBG revealed three potential sites at which a single point mutation would encode an Asp→Asn substitution and generate a consensus sequence for *N*-glycosylation (Van Baelen *et al.*, 1992). We have found one of these mutations in all the SHBG alleles of individuals from a family who are homozygous for an electrophoretic SHBG variant (Van Baelen *et al.*, 1992). When this point mutation was introduced into a human SHBG cDNA and the mutant cDNA was expressed in CHO cells, the additional consensus site for *N*-glycosylation was obviously utilized and the product was characterized by a super-heavy subunit which appears to be even larger than its counterpart in serum. However, the presence of this additional carbohydrate chain does not significantly alter the immunoreactivity or steroid-binding affinity of the protein, and is consistent

with the characteristics of this variant in serum samples (Van Baelen *et al.*, 1992). Our data also demonstrate that both the D327N SHBG mutant and the electrophoretic variant in serum comprise three electrophoretic isoforms that represent subunits that contain either one, two or three *N*-linked oligosaccharides. Furthermore, the Con A^+ forms of D327N SHBG clearly resolve into three distinct electrophoretic species, which suggests that these dimers that bind to Con A are composed exclusively of subunits containing only biantennary carbohydrate chains (Figure 4.6). In addition, the Con A^- fraction of D327N SHBG does not contain subunits completely lacking *N*-linked oligosaccharides as in the case for wild-type SHBG (Figure 4.6B), therefore it seems that the presence of an additional consensus site for *N*-glycosylation at position 327 increases the probability that the subunits acquire at least one *N*-linked oligosaccharide. It remains to be seen whether the same mutation is responsible for all forms of this type of triple-banded SHBG variant, which exists as a common phenotype within numerous ethnic groups throughout the world (Van Baelen *et al.*, 1992; Luckock and Cavalli-Sforza, 1983; Gershagen *et al.*, 1987; Khan *et al.*, 1985), but which appears not to be associated with any obvious pathological condition.

The migration of proteins in nondenaturing gels is influenced by molecular size, conformational shape and charge. However, it should be pointed out that since all the glycosylation mutants retain wild-type steroid-binding affinity and immunoreactivity, it is unlikely that there are any major perturbations in the conformation of the polypeptides. Furthermore, it is apparent that molecular size had very little influence on the migration patterns of the proteins in the gel since the SHBG glycosylation mutants with less carbohydrate content and thus lower molecular mass retained similar or lower mobilities than wild-type or D327N SHBG. Therefore, the different mobilities of the glycosylation mutants could be due to charge changes in the protein. For instance, the loss of the *O*-linked oligosaccharide resulted in the greatest alteration in electrophoretic mobility (compare wild-type SHBG versus T7A; or the mutant containing only the *O*-linked oligosaccharide versus the mutant completely lacking carbohydrate chains) and this suggests a

reduction in the negative charge of the protein. Furthermore, the differential effects manifested by the loss of either *N*-linked oligosaccharide independently, is illustrated by the different mobilities of the N351Q and N367Q mutants during PAGE, which also suggests a greater loss of negative charge in the N351Q mutant. Since both *N*-linked carbohydrate chains probably contain similar levels of sialic acid, the differential loss of negative charge could be due to the unmasking of positively charged residues that may interact with the carbohydrate chains at Asn^{351}. This suggests that the carbohydrate chain at Asn^{351} has a very different spatial orientation compared to the oligosaccharide at Asn^{367}, and evidence for this is provided by the gel filtration data which shows that the *N*-linked carbohydrate chains at these two positions have different effects on the hydrodynamic properties of SHBG. The significance of this is unknown but the specific orientation of an oligosaccharide chain and its potential interaction with the polypeptide could be important for the recognition of SHBG by various binding proteins or receptors in the plasma membrane.

In conclusion, the carbohydrates associated with human SHBG do not influence its steroid-binding properties and are not essential for its biosynthesis as a homodimer. This is important because it suggests that functional forms of SHBG may be produced in bacteria which do not glycosylate proteins. Carbohydrates influence the half-life of glycoproteins in the blood (Galway *et al.*, 1990; Hossner and Billiar, 1981; Braunstein *et al.*, 1972; Ain *et al.*, 1987), and the biological properties of the mutants produced as a result of these studies could be exploited in an animal model. Since the carbohydrate components of some plasma glycoproteins are important for interaction with their respective membrane-bound receptors (Combarnous, 1992), and for initiating signal transduction at the cell membrane (Keene *et al.*, 1989; Sairam and Bhargavi, 1985; Combarnous, 1992), the SHBG mutants we have created will be invaluable for characterizing SHBG receptors that have been identified in the plasma membranes of sex steroid-responsive tissues (Porto *et al.*, 1992b; Strel'chyonok *et al.*, 1984b; Krupenko *et al.*, 1990; Hryb *et al.*, 1985; Porto *et al.*, 1992a).

CHAPTER 5

LOCALIZATION OF THE STEROID-BINDING DOMAIN IN SHBG

5.1 Introduction

Studies of the human SHBG and rat ABP steroid-binding sites using affinity labels have produced inconsistent results (Grenot *et al.*, 1988; Danzo *et al.*, 1991; Namkung *et al.*, 1990; Hammond *et al.*, 1987; Petra *et al.*, 1988; Khan and Rosner, 1990; Grenot *et al.*, 1992), and have raised questions as to whether amino acids that interact with steroid ligands are confined to a discrete region (Grenot *et al.*, 1988; Danzo *et al.*, 1991; Namkung *et al.*, 1990; Grenot *et al.*, 1992) or are dispersed throughout the molecule (Hammond *et al.*, 1987; Petra *et al.*, 1988; Khan and Rosner, 1990). To resolve this issue, human SHBG/rat ABP chimeras were created based on the fact that the steroid-binding affinities of human SHBG and rat ABP are quite distinct (Tindall and Means, 1980; Westphal, 1986). Thus it was predicted that the chimeras would either retain or lose human steroid-binding characteristics depending on the content and location of human sequences.

The primary structure of human SHBG was compared with sex steroid-binding proteins in other mammalian species (Hammond, 1993). This revealed a sequence of poorly conserved polar amino acids adjacent to Met^{139} (Figure 5.1), which has been affinity-labeled in human SHBG (Grenot *et al.*, 1988; Grenot *et al.*, 1992). Therefore, this residue was substituted by tryptophan (M139W) to determine if this would affect the steroid-binding affinity of SHBG. In addition, Ser^{133} and His^{136} in human SHBG were also individually converted to Asp^{133} (S133D) and Gln^{136} (H136Q) respectively (Figure 5.2C), because these residues are conserved in the sex steroid-binding proteins of the other species (Figure 5.1), which bind steroids with less affinity than human SHBG. These recombinant proteins were expressed in CHO cells, and their steroid-binding and immunological properties were analyzed.

5.2 Results

5.2.1 Primary Structure of Human SHBG/Rat ABP Chimeras

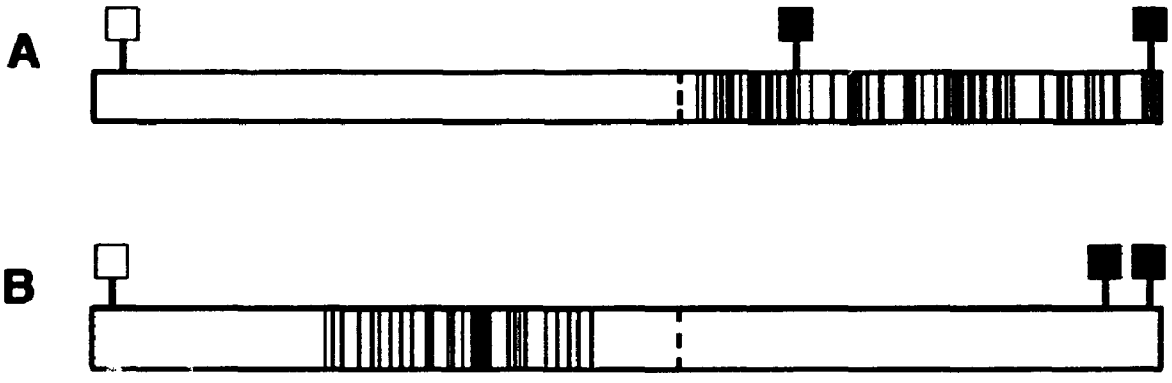
Human SHBG and rat ABP both comprise 373 amino acids (Figure 1.3) and exhibit 68% sequence identity (Reventos *et al.*, 1988). Their cDNAs contain a conserved internal *Eco*RI site, which was utilized to create a hybrid cDNA (see

Figure 5.1 Phylogenetic comparison of SHBG-related protein sequences between residues 131-149. Single letter amino acid code is used and identity to human SHBG is indicated by a *dash*.

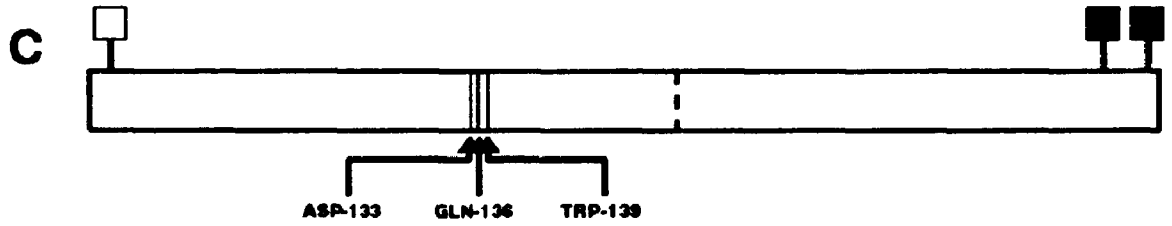
SPECIES	RESIDUES NUMBERED AS IN HUMAN SHBG																	
	131							139										149
HUMAN	L	T	S	K	R	H	P	I	M	R	I	A	L	G	G	L	L	F	P
RAT	-	A	D	H	P	Q	L	S	-	-	-	-	-	-	-	-	-	L	-
MOUSE	-	A	D	H	S	Q	R	S	-	-	-	-	-	-	-	-	-	L	-
RABBIT	-	H	D	-	P	Q	-	V	-	K	L	-	V	-	-	-	-	-	-
SHEEP	-	A	N	N	S	Q	L	-	-	-	-	-	-	-	-	-	-	-	-

Figure 5.2 Schematic of the human SHBG/rat ABP chimeras and the human SHBG mutants. The proteins comprise 373 amino acids and *vertical lines* indicate positions of rat ABP-specific amino acids which account for 35% of the residues located between positions 205-373 in chimera A (A) and 30% of the residues between positions 77-176 in chimera B (B). The positions of the three independent amino acid substitutions in human SHBG introduced by site-directed mutagenesis are also designated by *vertical lines* (C). The conserved *Eco*RI site in the human SHBG and rat ABP cDNA sequences encompasses the codons for Glu^{205} and Phe^{206} and its relative position is shown as a *broken vertical line*. *Closed boxes* indicate the locations of *N*-linked oligosaccharide chains at Asn^{351} and Asn^{367} in chimera B (B) and the human SHBG mutants (C), and Asn^{244} and Asn^{367} in chimera A (A). The *O*-linked oligosaccharide at Thr^{7} is shown as an *open box*.

Human SHBG/Rat ABP Chimeras



Human SHBG Mutants



2.2.3) encoding a human SHBG/rat ABP chimera with residues 1-205 encoded by the human cDNA and residues 206-373 encoded by the rat cDNA (Figure 5.2A, chimera A). A second chimera was also created by substituting a portion of the human SHBG cDNA encoding amino acids 77-176 and which encompasses Met^{139}, with the analogous rat ABP coding sequences (Figure 5.2B, chimera B) by using a cDNA insert that was generated by PCR (see 2.2.4). Both hybrid cDNAs were expressed in CHO cells and the culture medium containing the resultant chimeras was analyzed.

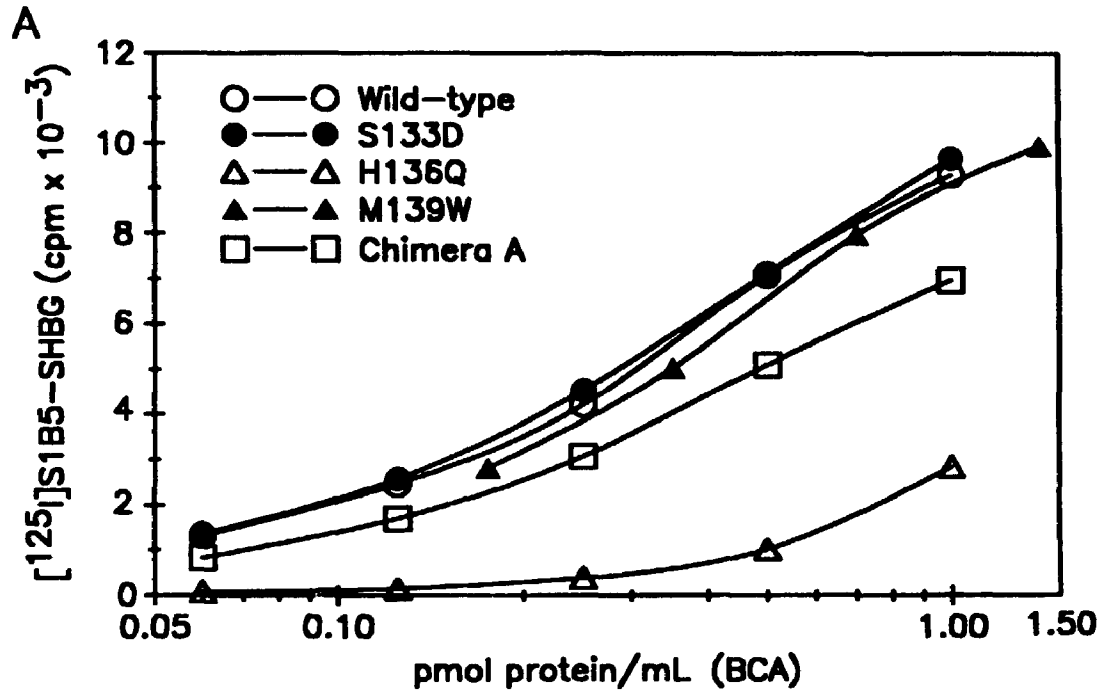
5.2.2 Immunochemical Detection of Human SHBG/Rat ABP Chimeras

Initially, culture medium was examined for the presence of the chimeras using an IRMA (see 2.9.1) that relies on the use a monoclonal antibody against human SHBG, which recognizes a species-specific conformational epitope (Hammond and Robinson, 1984). Chimera A (Figure 5.2A) cross-reacts significantly in this IRMA (Figure 5.3A), but chimera B could not be detected even after a 10-fold concentration of the culture medium. By contrast, chimera A was poorly recognized by a rabbit polyclonal antiserum against human SHBG in a conventional RIA (Figure 5.3B), which obviously reflects a loss of epitopes within the *C*-terminal portion of the molecule.

5.2.3 Steroid-binding Properties of Human SHBG/Rat ABP Chimeras

The apparent dissociation-rate (see 2.11.2) of [^{3}H]DHT from the steroid-binding sites of chimera A and wild-type SHBG were both < 5% after 10 minutes of DCC exposure (Figure 5.4A), and their affinities for DHT (k_d=0.4 nM) as measured by Scatchard analysis (see 2.11.3) were identical (Figure 5.4B). Furthermore, chimera A bound other steroid ligands with a relative affinity that was similar to wild-type SHBG (Table 5.1). This is remarkable because 35% of the residues in the *C*-terminal portion of chimera A (residues 206-373) are specific for rat ABP (Figure 5.2A). However, chimera B which contains a series of 100 residues of which 30% are specific for rat ABP (Figure 5.2B) displayed limited steroid-binding capacity (0.44 nM) that was only detectable when 10-fold concentrated culture medium was

Figure 5.3 Analyses of wild-type SHBG, the human SHBG mutants and chimera A by (A) an immunoradiometric assay (IRMA) and (B) a radioimmunoassay (RIA). The IRMA utilizes a mouse monoclonal antibody specific for human SHBG (see 2.9.1) and the RIA uses a rabbit polyclonal antiserum against human SHBG (see 2.9.2). A steroid-binding capacity assay (BCA) was used to define the amounts of protein analyzed in each assay (see 2.11.1). Each *data point* represents the mean value of duplicate measurements.


A
12
10
8
6
4
2
0
[125I]S1B5-SHBG (cpm x 10−3)
Wild-type
S133D
H136Q
M139W
Chimera A
0.05
0.10
1.00
1.50
pmol protein/mL (BCA)

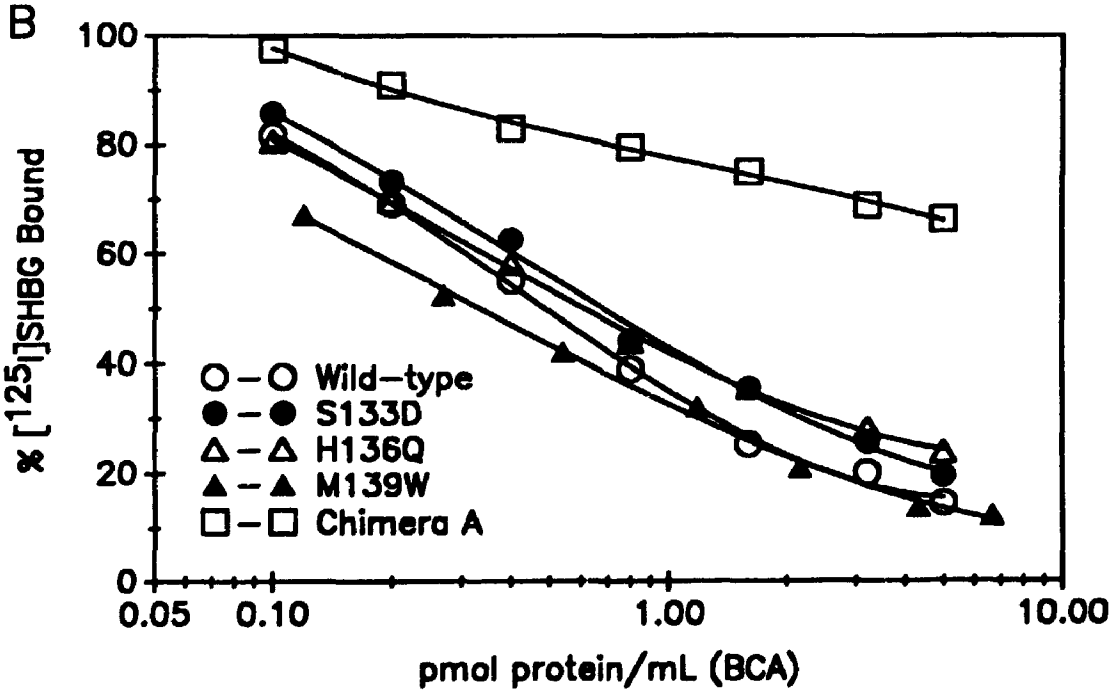
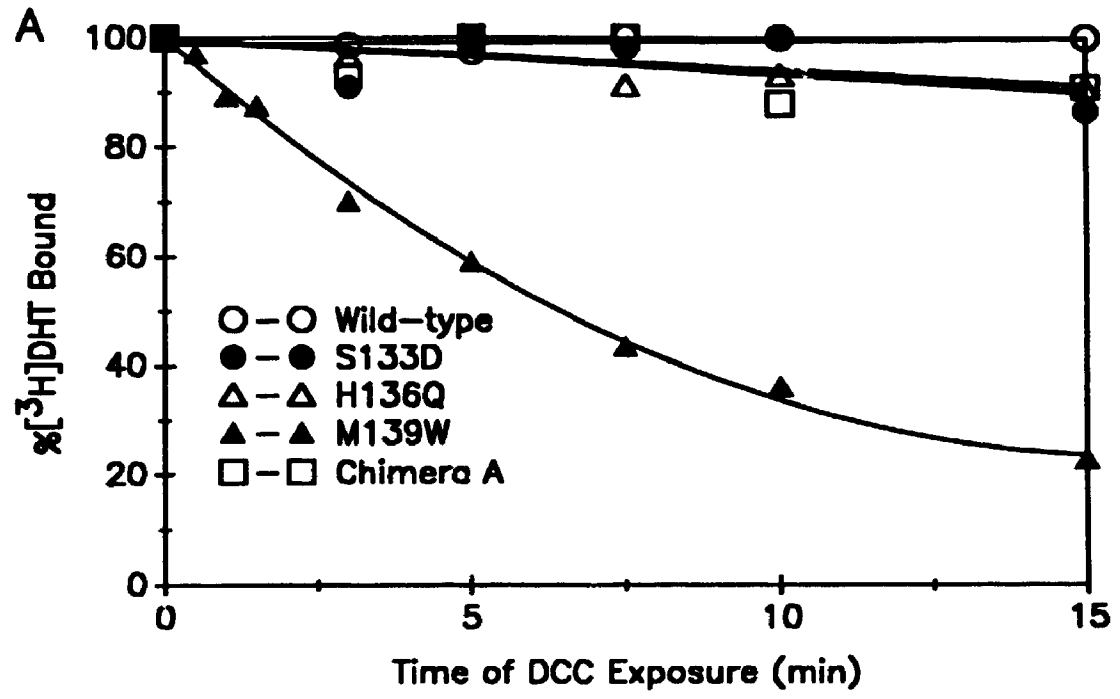

B
100
80
60
40
20
0
% [125I]SHBG Bound
Wild-type
S133D
H136Q
M139W
Chimera A
0.05
0.10
1.00
10.00
pmol protein/mL (BCA)

Figure 5.4 Measurements of the apparent dissociation rate (A) and Scatchard analysis (B) of wild-type SHBG, the human SHBG mutants and chimera A. Measurements for both assays were taken at 0°C using [^{3}H]DHT as the labeled ligand and DCC to separate bound and free steroid. Each *data point* in both graphs represents the mean value of triplicate measurements. The apparent dissociation rate was 67% for M139W SHBG, and < 5% for the remaining proteins (A). Dissociation constants (k_d) for the proteins are as follows: wild-type SHBG, k_d=0.40 nM; chimera A, k_d=0.40 nM; S133D SHBG, k_d=0.34 nM; H136Q SHBG, k_d=0.35 nM; M139W SHBG, k_d=2.08 nM (B).


A
100
80
60
40
20
0
%[3H]DHT Bound
O—O Wild-type
●—● S133D
△—△ H136Q
▲—▲ M139W
□—□ Chimera A
0
5
10
15
Time of DCC Exposure (min)

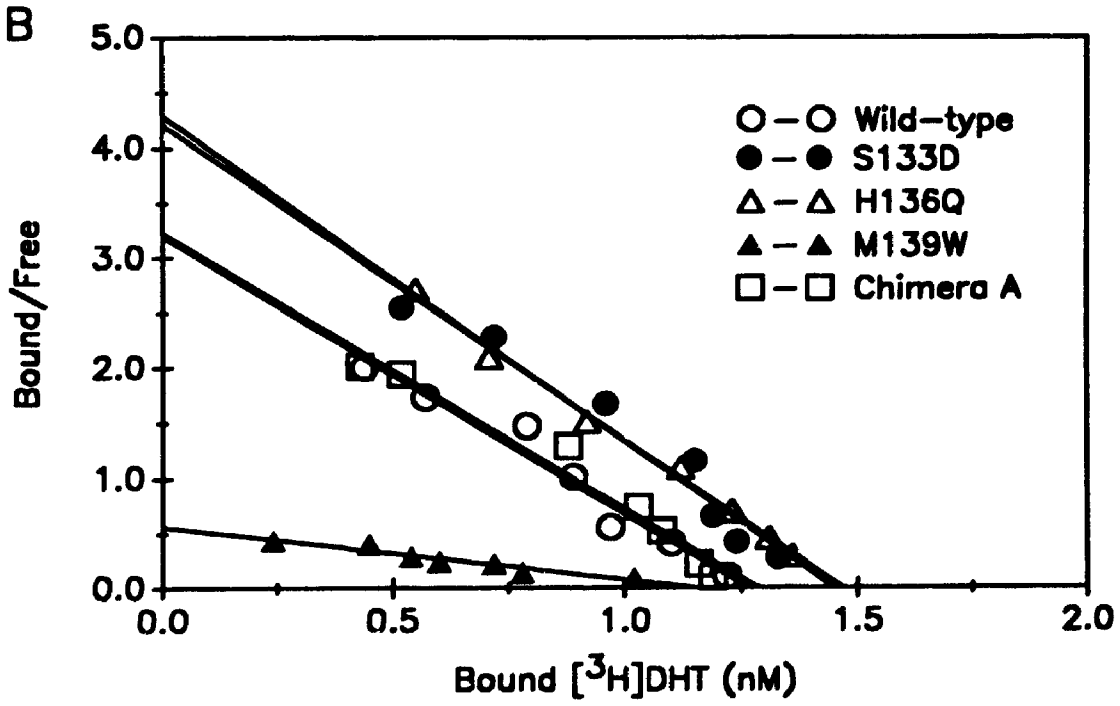

B
5.0
4.0
3.0
2.0
1.0
0.0
Bound/Free
O—O Wild-type
●—● S133D
△—△ H136Q
▲—▲ M139W
□—□ Chimera A
0.0
0.5
1.0
1.5
2.0
Bound [3H]DHT (nM)

Table 5.1 The relative binding affinities of wild-type SHBG and chimera A for different steroids. The relative binding affinity of SHBG for each steroid was defined as the amount of DHT required to displace 50% of the labeled ligand, divided by the amount of other competitor steroids to displace 50% of the labeled ligand. Measurements were taken at 0°C using [^{3}H]DHT as the labeled ligand and DCC to separate bound and free steroid.

STEROID	RELATIVE BINDING AFFINITY	
	WILD-TYPE SHBG	CHIMERA A
5α-Dihydrotestosterone	100	100
Testosterone	20	18
17β-Estradiol	7	5

measured in an assay where bound and free steroid were separated with DCC for only 5 min. It should be noted that steroid-binding activity was undetectable in 10-fold concentrated conditioned medium from untransfected CHO cells and used as a control.

5.2.4 Electrophoretic Analysis of Human SHBG/Rat ABP Chimeras

Both chimeras were subjected to denaturing-PAGE and western blotting, and could be detected using a rabbit polyclonal antiserum against human SHBG. Chimera A was composed of subunits that migrated with slightly lower mobilities than the typical heavy and light subunits associated with wild-type SHBG (Figure 5.5A). By contrast, the medium containing chimera B was concentrated 10-fold for western blot analysis and it displayed the same subunit size heterogeneity and mobility as the heavy and light subunits of concentrated wild-type SHBG (Figure 5.5B).

5.2.5 Immunoactivity of Human SHBG Mutants

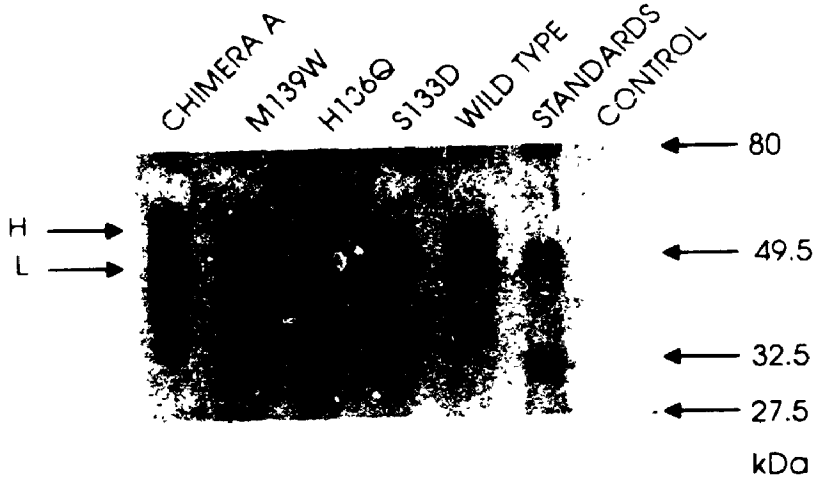
The M139W and S133D mutants were as immunoreactive as wild-type human SHBG, while the H136Q mutant reacted poorly in an IRMA (Figure 5.3A). However, all three mutants displayed similar cross-reactivity with wild-type SHBG in a conventional RIA (Figure 5.3B), which compares the ability of unlabeled mutants to compete with [^{125}I]SHBG for a rabbit polyclonal antiserum against human SHBG.

5.2.6 Steroid-binding Properties of Human SHBG Mutants

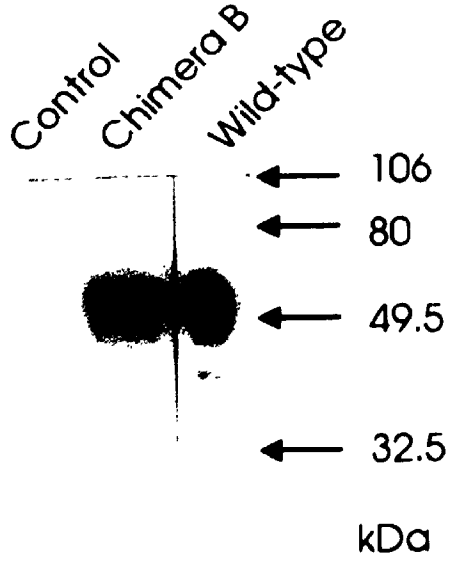
The M139W mutant displayed a marked reduction in steroid-binding affinity for [^{3}H]DHT, as assessed by a dissociation rate of 67% (Figure 5.4A) and a k_d=2.08 nM as measured by Scatchard analysis (Figure 5.4B). However, the steroid-binding affinities of the S133D (k_d=0.34 nM) and H136Q (k_d=0.35 nM) SHBG mutants were indistinguishable from those of wild-type human SHBG (k_d=0.40 nM) and their [^{3}H]DHT dissociation rates were < 5% (Figure 5.4).

Figure 5.5 Western blots of wild-type SHBG, the human SHBG mutants and the chimeras. (A) Culture medium samples of wild-type SHBG, the human SHBG mutants, and chimera A were subjected to denaturing-PAGE (7.5% resolving gel) prior to western blotting. Conditioned medium from untransfected CHO cells was examined as a control. The positions of the heavy (*H*) and light (*L*) subunits of the proteins are shown on the *left*. (B) Culture medium samples of wild-type SHBG and chimera B were concentrated 10-fold and an aliquot of each sample was subjected to denaturing-PAGE (10% resolving gel) prior to western blotting. Concentrated conditioned medium from untransfected CHO cells was examined as a control. In both (A) and (B), a rabbit polyclonal antiserum against human SHBG was used as primary antibody for western blot analysis and the positions of size standards are shown on the *right*.

A


CHIMERA A
M139W
H136Q
S133D
WILD TYPE
STANDARDS
CONTROL
H
L
80
49.5
32.5
27.5
kDa

B


Control
Chimera B
Wild-type
106
80
49.5
32.5
kDa

5.2.7 Electrophoretic Analysis of Human SHBG Mutants

The subunit composition of the human SHBG mutants was assessed under denaturing conditions by western blotting, and they displayed the same typical size heterogeneity and migrated with identical mobilities as wild-type human SHBG (Figure 5.5A).

5.3 Discussion

The expression and analyses of the human SHBG/rat ABP chimeras clearly demonstrated that the amino acids required for the high affinity steroid-binding characteristics of human SHBG are located within residues 1-205. This was supported by the difficulty in reliably measuring the steroid-binding activity of chimera B, despite its detection by western analysis. In this context, it is important to note that the level of detectable protein on the blot may have been an underestimation since chimera B probably lacked many epitopes recognized by the polyclonal antiserum against human SHBG used for this purpose. In addition, proteins with relatively low affinity for steroid ($k_d > 10^{-9}$ M) are difficult to detect using charcoal separation because of the rapid dissociation of steroid from the protein. Therefore, this may explain the discrepancy between the very low steroid-binding capacity of chimera B, with respect to its protein level detected on a western blot. It should be noted that these data do not exclude the possibility that residues in the *C*-terminal portion of human SHBG, which are conserved in rat ABP, could contribute to the appropriate folding and dimerization of SHBG that may be required for the formation of its steroid-binding site.

The M139W SHBG mutant had a 5-fold lower affinity for DHT than wild-type SHBG, while substitutions at residues 133 and 136 had no effect on steroid binding. The M139W SHBG mutant retained wild-type immunoreactivity and therefore the decreased steroid-binding affinity of this mutant is unlikely due to a gross alteration in protein structure. Subsequent studies by others in which Met^{139} in human SHBG and rat ABP has been replaced by other amino acids, have resulted in mutants with reduced affinity for DHT (Sui *et al.*, 1992; Joseph and Lawrence, 1993). However,

one substitution (Met^{139}→Leu) failed to affect steroid binding and it was suggested that the hydrophobic side chain of Met^{139} can be replaced by the leucine side chain (Sui *et al.*, 1992). Unfortunately in this latter study, 5% FBS was present in the culture medium and the steroid-binding properties of these mutants may be difficult to interpret (Sui *et al.*, 1992). Nevertheless, it appears that Met^{139} is an important component of the steroid-binding domain in SHBG from all species.

The His→Gln substitution at position 136 appears to disrupt the epitope recognized by the monoclonal antibody used in the IRMA, without influencing steroid-binding or the overall conformation of the molecule as assessed by its immunochemical behaviour in the RIA, i.e., it exhibits a displacement curve that is parallel to wild-type SHBG. In addition, His^{136} resides in a span of very poorly conserved amino acids and this certainly explains why this monoclonal antibody is specific for human SHBG. Furthermore, chimera A reacted well in the IRMA, while chimera B containing rat ABP-specific residues between positions 77-176 did not. This confirms that the epitope recognized by the monoclonal antibody resides in the *N*-terminal portion of human SHBG, and other mutations in this region would might therefore delineate other amino acids that form part of this epitope.

Chimera A containing rat ABP-specific amino acids within residues 206-373, is composed of heavy and light subunits that are larger than the typical subunit isoforms for human SHBG when assessed by electrophoresis under denaturing conditions. Since rat ABP contains an *N*-linked carbohydrate chain at Asn^{244} in place of Asn^{351}, this supports the theory that the number and/or position of *N*-glycosylation sites within the protein may influence the processing of particular carbohydrate chains. In addition, the presence of an oligosaccharide at Asn^{244} does not interfere with the steroid-binding properties of chimera A, which again demonstrates that carbohydrates play no role in this process.

In summary, the analyses of chimeric sex steroid-binding proteins has localized the steroid-binding domain to the *N*-terminal half of human SHBG in a region that

includes Met[139]. More importantly, these experiments demonstrated that amino acid substitutions can be introduced into SHBG which specifically disrupt steroid binding without disturbing the conformation of the entire protein. Therefore, in the absence of any tertiary structure information, site-directed mutagenesis appears to be a useful means of identifying amino acids that may be specifically involved in steroid binding and possibly the dimerization of SHBG.

CHAPTER 6

SHBG STEROID-BINDING AND DIMERIZATION DOMAINS PARTIALLY OVERLAP

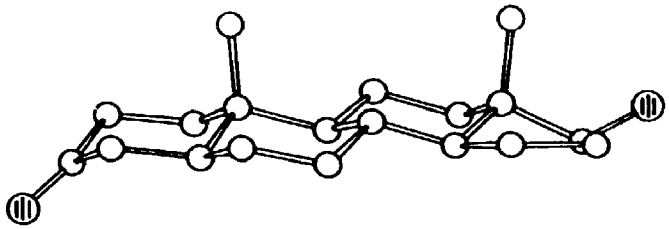
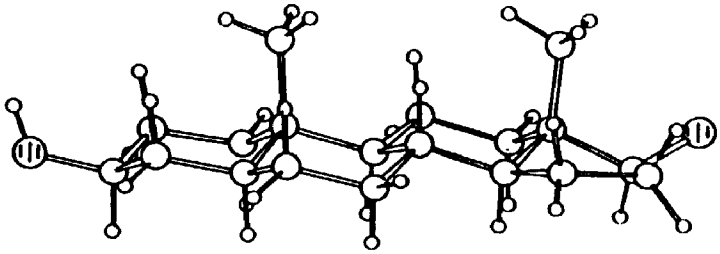
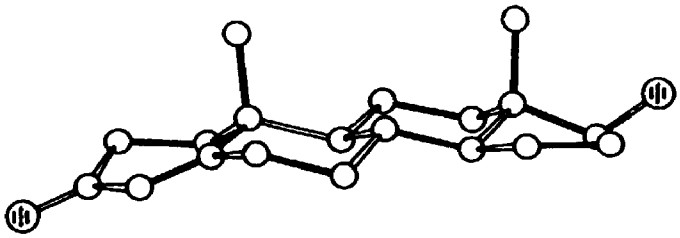
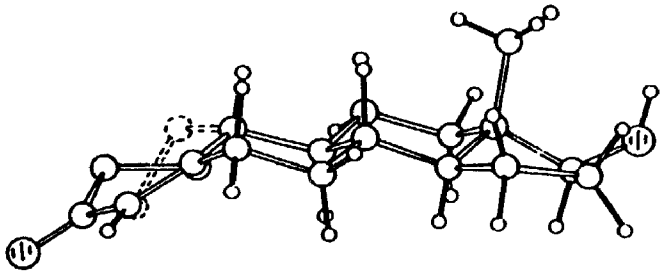
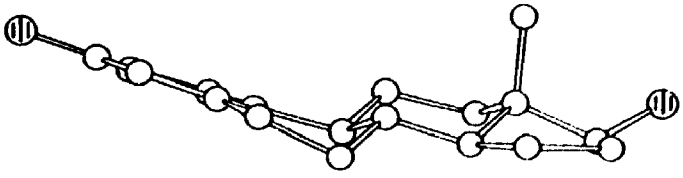
6.1 Introduction

The requirements for steroid binding to SHBG have been studied in great detail (Cunningham *et al.*, 1981), but information about the orientation of ligands in the steroid-binding site and its topography is limited. Like its counterparts in other species, human SHBG binds DHT with highest affinity (Westphal, 1986), but these proteins also interact with several other steroids with functional groups that undoubtedly make contact with specific amino acids in their binding sites. Thus, it was proposed that different steroid ligands, with unique structural properties (Table 6.1), may be utilized as molecular probes to evaluate subtle changes in steroid-binding specificity that might occur as a result of specific amino acid substitutions. Moreover, this approach might reveal whether certain residues within the steroid-binding site make contact with particular functional groups or structural features on a given steroid ligand.

Dimerization of SHBG is 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), and this suggests that an interaction with divalent cation, as well as occupancy of the steroid-binding site, may help maintain its structural integrity. Studies using affinity-labeling (Grenot *et al.*, 1992; Grenot *et al.*, 1988) have indicated that Met^{139} in human SHBG is probably important for interaction with the steroid B ring and we (Chapter 5) and others (Sui *et al.*, 1992), have also confirmed this using site-directed mutagenesis. In addition, the steroid-binding properties of human SHBG/rat ABP chimeras have suggested that residues important for high affinity steroid binding are situated within the *N*-terminal 205 amino acids of human SHBG (Chapter 5). Therefore, individual amino acid substitutions were introduced in a region encompassing Met^{139}, and at other locations in the SHBG molecule, in order to identify residues that influence its ability to bind steroid and/or dimerize.

Since carbohydrates do not interact with steroid ligands and are not required

Table 6.1 The relative binding affinities of SHBG for the steroid ligands used as molecular probes of the SHBG steroid-binding site. The carbon atom backbone in each conformation diagram is represented by *large open circles* and oxygen atoms are indicated as *hatched circles*. Hydrogen atoms are shown as *small open circles* in the diagrams of 5αA and 19norT. Diagrams of steroid conformations were derived from Duax and Norton, 1975. 5α-DHT, 5α-dihydrotestosterone; 5αA, 5α-androstane-3ß,17ß-diol; T, testosterone; 19norT, 19-nor-testosterone; E_2, 17ß-estradiol.

STEROID	STRUCTURE	RELATIVE BINDING AFFINITY
5α-DHT		100
5αA		30
T		20
19norT		0.5
E_2		5

for the formation of its steroid-binding site, it was considered that the expression of human SHBG in *E. coli* might provide another means of studying the functional regions of human SHBG. Attempts to define the minimal steroid-binding domain of rat ABP by expressing deletion mutants in mammalian cells have been unsuccessful (Joseph and Lawrence, 1993), and this may be explained by defects in production and/or secretion due to inappropriate folding or subunit association in mammalian cells. To circumvent these problems, and to identify an expression system capable of synthesizing large amounts of recombinant SHBG with steroid-binding activity for detailed functional and physicochemical analyses, human SHBG and various SHBG deletion mutants were produced as glutathione S-transferase (GST) fusion proteins in *E. coli*.

6.2 Rationale For Mutations

A region immediately *N*-terminal of Met^{139}, which is very poorly conserved in sex steroid-binding proteins of various species and contains predominantly hydrophilic amino acids (Hammond, 1993), was targeted for mutagenesis. The choice of amino acid substitutions was based primarily on a phylogenetic comparison of SHBG/ABP sequences (Figure 5.1) because it was thought that residues in this location could be responsible for the differences in steroid-binding affinity between species (Westphal, 1986). Furthermore, species differences in amino acid sequence that have appeared during evolution are obviously tolerated, therefore these substitutions would probably not perturb the structure of human SHBG to the extent that it might eliminate secretion.

A highly conserved region immediately *C*-terminal of Met^{139} containing predominantly hydrophobic residues (Figure 5.1), was also subjected to mutagenesis because it was reasoned that these residues might form a hydrophobic binding pocket for a steroid ligand. 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 of these mutants, more conservative substitutions were introduced.

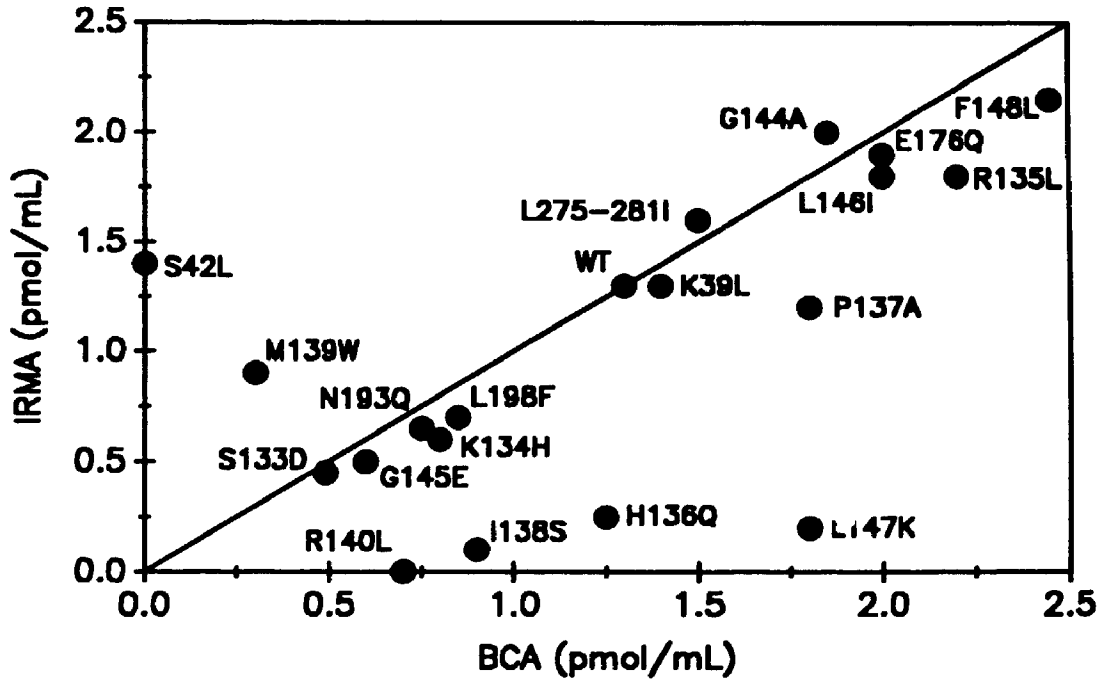
Amino acids at positions 176, 193, and 198 in human SHBG were substituted for residues that were conserved in the sex steroid-binding proteins of other species. In addition, substitutions were created at residues 39 and 42 in human SHBG that reside in a well conserved, conspicuous stretch of polar amino acids within residues 35-43 (Figure 1.3). Another mutant was created in which leucines at positions 275, 277, 279 and 281 (Figure 1.3) were each substituted with isoleucine because this region has been proposed to contribute to steroid binding and/or dimerization (Walsh *et al.*, 1986; Petra, 1991). Finally, the cysteines that compose the two intramolecular disulphide bridges (Cys^{164}-Cys^{188}, Cys^{333}-Cys^{361}) were mutated in pairs to serines to disrupt each disulphide linkage independently, and a Cys^{333}→Stop mutation was created to truncate SHBG at the *C*-terminus. The substitution of the cysteines might determine if the loss of secondary structure would adversely affect steroid-binding or dimerization. All recombinant SHBG mutants were expressed in CHO cells.

6.3 Results

6.3.1 Detection of Human SHBG Mutants Expressed in CHO Cells

A steroid-binding capacity assay (see 2.11.1) and an IRMA (see 2.9.1) were used to measure the amount of each SHBG mutant in the culture medium. As in the case for wild-type SHBG, the majority of the mutants were detected in similar amounts using both assays as demonstrated by their position on the graph relative to the line that represents equal concentrations of SHBG as measured by both assays (Figure 6.1). Four mutants (H136Q, I138S, R140L, and L147K) displayed lower immunoactivity compared to wild-type SHBG but retained the ability to bind [^{3}H]DHT. Conversely, two mutants (M139W and S42L) had reduced or undetectable steroid-binding capacities with respect to their immunoreactivity. Only three of the SHBG mutants with single amino acid substitutions (A142E, G144E, and L146K) were not detectable by either assay and were not secreted in sufficient amounts for further analysis, as determined by western analysis. In addition, the mutants with disrupted disulphide bridges and the SHBG mutant truncated at the *C*-terminus were not secreted and were not analyzed further.

Figure 6.1 Comparison of the concentrations of wild-type SHBG and the SHBG mutants secreted into the culture medium, as measured by an immunoradiometric assay (IRMA) and a steroid-binding capacity assay (BCA). The line on the graph represents the situation where the steroid-binding capacity of SHBG in the sample is equivalent to its immunochemical-determined concentration. WT, wild-type SHBG.


2.5
2.0
1.5
1.0
0.5
0.0
IRMA (pmol/mL)
0.0
0.5
1.0
1.5
2.0
2.5
BCA (pmol/mL)
G144A
F148L
E176Q
R135L
L146I
L275–281I
WT
K39L
S42L
P137A
M139W
N193Q
L198F
K134H
S133D
G145E
H136Q
I138S
R140L

6.3.2 Steroid-binding Properties of Human SHBG Mutants

Unlike M139W and S42L, the other SHBG mutants bound [^{3}H]DHT with affinities that are indistinguishable from wild-type SHBG based on the apparent dissociation rate of labeled ligand from their binding sites (< 5%) (see 2.11.2). The relative steroid-binding affinities of the SHBG mutants were compared initially to wild-type SHBG in a competition assay (see 2.11.4) using predetermined amounts of DHT, testosterone (T), 17ß-estradiol (E_2), 5α-androstane-3ß,17ß-diol (5αA), androst-5-ene-3ß,17ß-diol (Δ5A) and 19-nor-testosterone (19norT) that displace 50% of the tracer from wild-type SHBG (Table 6.1). Mutants with affinities for ligands, that differed by more than 3 standard deviations (SD) of the mean values obtained for wild-type SHBG, were analyzed further with a broad concentration of appropriate steroids to generate complete competition curves. While these mutants all bound T and 19norT with the same affinity as wild-type SHBG (Table 6.2), 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 SHBG mutant (Table 6.2, Figure 6.2). Conversely, three of these mutants displayed an increased affinity for E_2 and this was best demonstrated by the I138S and R135L SHBG mutants (Table 6.2, Figure 6.2). Furthermore, the R135L mutant also had an approximately 2-fold increase in affinity for C19 steroids with a 3ß-OH group (5αA and Δ5A), when compared to wild-type SHBG (Table 6.2, Figure 6.3). It should be noted that when unlabeled DHT was used as competitor, the competition curves for these mutants were superimposable with those obtained for wild-type SHBG (Figures 6.2, 6.3), and this indicates that their affinities for DHT are essentially identical. Substitutions in the region immediately *C*-terminal to Met^{139} (residues 140-148) and at residues 176, 193, 198 had no effect on the steroid-binding properties of SHBG. Furthermore, the L275-281I SHBG mutant also bound steroids with wild-type characteristics (Table 6.2). In a separate experiment, the K39L SHBG bound steroid ligands (DHT and E_2) with the same affinity as wild-type SHBG, as depicted in Figure 6.2.

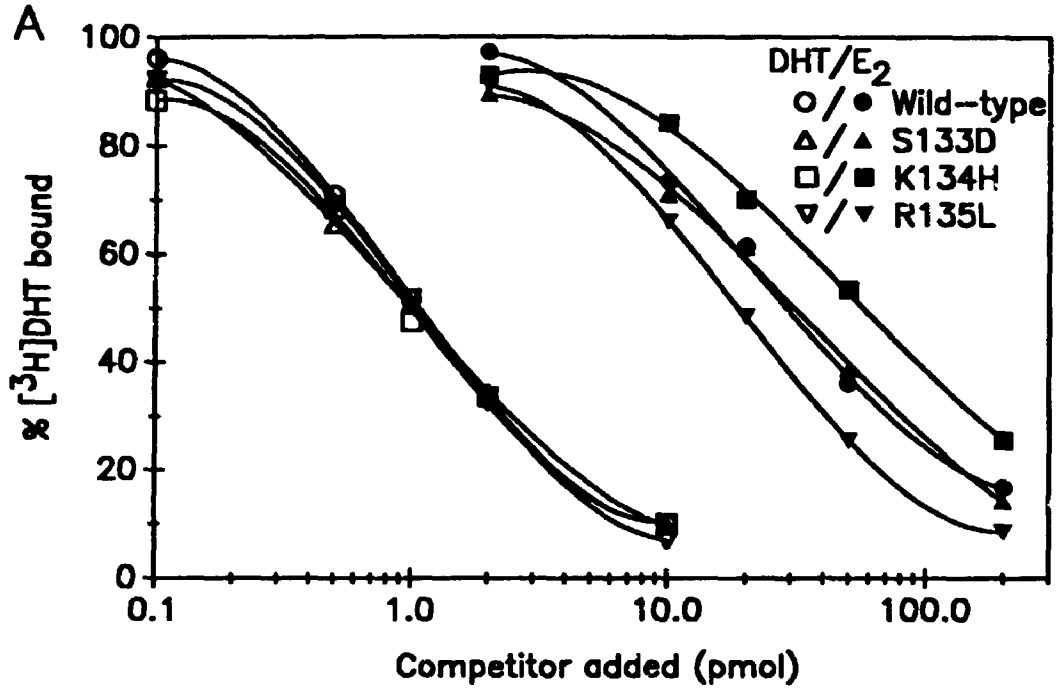
The same methods were used to examine the steroid-binding characteristics of

Table 6.2 Affinities of steroid ligands for SHBG mutants relative to wild-type SHBG. The SHBG mutants were screened for their relative steroid-binding affinities using predetermined amounts of the 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. The affinity of an SHBG mutant for a specific steroid was determined to be equal to wild-type SHBG if the measurement was within the mean ± 3 SD of the wild-type value. Values in *parentheses* are derived from complete competition curves in Figures 6.2, 6.3, and Table 6.3. T, testosterone; 5αA, 5α-androstane-3ß,17ß-diol; Δ5A, androst-5-ene-3ß,17ß-diol; 19norT, 19-nor-testosterone; E_2, 17ß-estradiol.

SHBG MUTANTS	RELATIVE BINDING AFFINITY FOR STEROID LIGANDS				
	T	5αA	Δ5A	19norT	E_2
S133D	=	=	=	=	=
K134H	=	=	=	=	↓ (2x)
R135L	=	↑ (1.6x)	↑ (1.8x)	=	↑ (2x)
H136Q	=	=	=	=	↓ (1.3x)
P137A	=	=	=	=	↑ (1.3x)
I138S	=	=	=	=	↑ (2.5x)
M139W	↓ (5x)	↓ (5x)	↓ (2x)	=	↓ (5x)
R140L	=	=	=	=	=
G144A	=	=	=	=	=
G145E	=	=	=	=	=
L146I	=	=	=	=	=
L147K	=	=	=	=	=
F148L	=	=	=	=	=
E176Q	=	=	=	=	=
N193Q	=	=	=	=	=
L198F	=	=	=	=	=
L275-281I	=	=	=	=	=

= Equal affinity to wild-type human SHBG (mean ± 3 SD)
↑ Increased affinity relative to wild-type human SHBG
↓ Decreased affinity relative to wild-type human SHBG

Figure 6.2 Affinities of wild-type SHBG and SHBG mutants 133-138 for 17β-estradiol (E_2) relative to 5α-dihydrotestosterone (DHT). Competition curves were generated by incubating the SHBG samples with [^{3}H]DHT and increasing amounts of 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.


A
100
80
60
40
20
0
% [3H]DHT bound
DHT/E2
Wild-type
S133D
K134H
R135L
0.1
1.0
10.0
100.0
Competitor added (pmol)

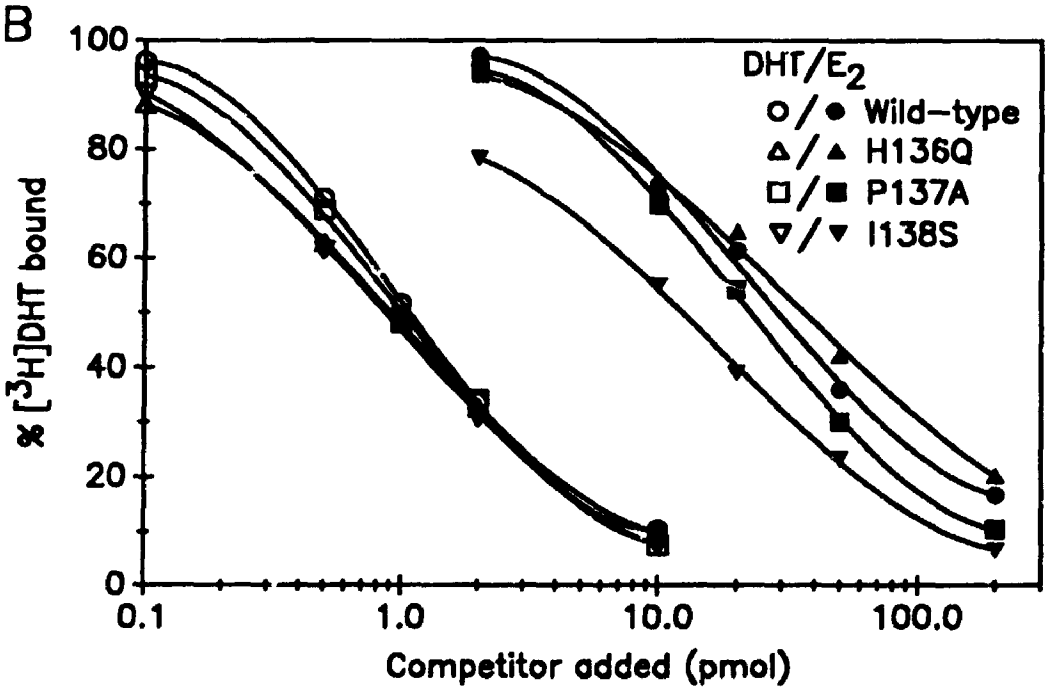
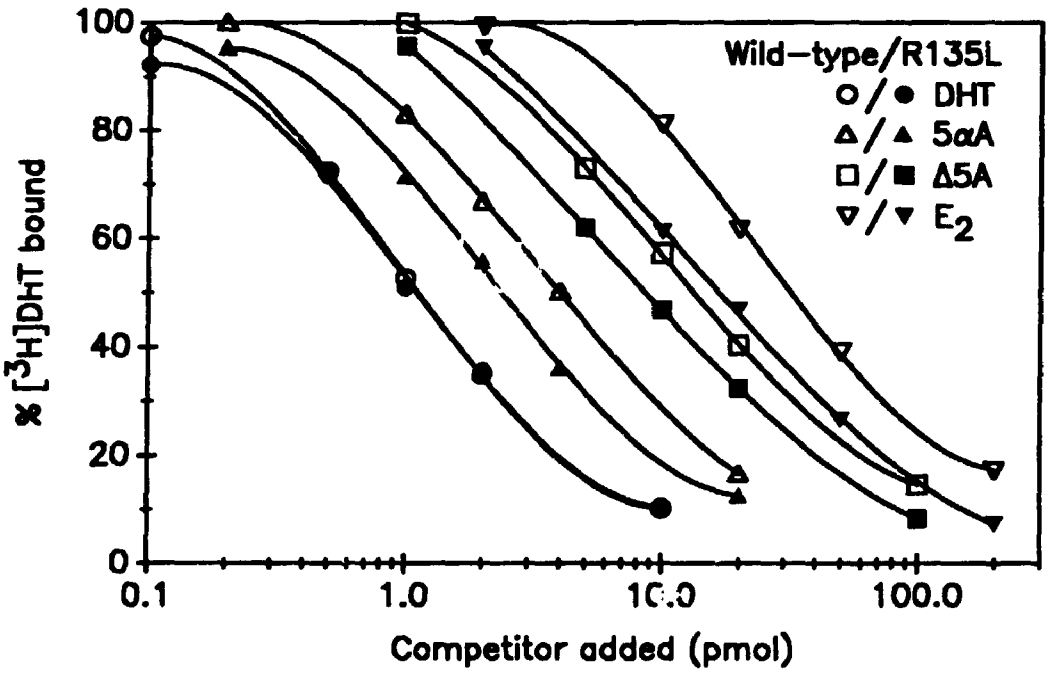

B
100
80
60
40
20
0
% [3H]DHT bound
DHT/E2
Wild-type
H136Q
P137A
I138S
0.1
1.0
10.0
100.0
Competitor added (pmol)

Figure 6.3 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 incubating with [^{3}H]DHT and increasing amounts of unlabeled DHT, 5α-androstane-3ß,17ß-diol (5αA), androst-5-ene-3ß,17ß-diol (Δ5A), or 17ß-estradiol (E_2).


Wild–type/R135L
○/● DHT
△/▲ 5αA
□/■ Δ5A
▽/▼ E2
% [3H]DHT bound
100
80
60
40
20
0
0.1
1.0
10.0
100.0
Competitor added (pmol)

the M139W mutant, which binds DHT with 5-fold lower affinity than wild-type SHBG (Chapter 5), except that 1 nM M139W SHBG 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 as a control in these experiments. When compared to the affinity of wild-type SHBG for various ligands, the M139W mutant displayed 2.5-fold and 5-fold greater affinities for Δ5A and 19norT relative to DHT, respectively, while its affinities for T, 5αA and E_2, relative to DHT, were very similar to those associated with the wild-type protein (Table 6.3). The S42L mutant which was recognized by the IRMA, did not exhibit any detectable steroid-binding affinity for [^{3}H]DHT as determined by dissociation rate analysis (see 2.11.2). Although it is conceivable that S42L SHBG could bind other steroid ligands, its binding properties were not examined further.

6.3.3 Influence of Steroid Ligands on Dimerization

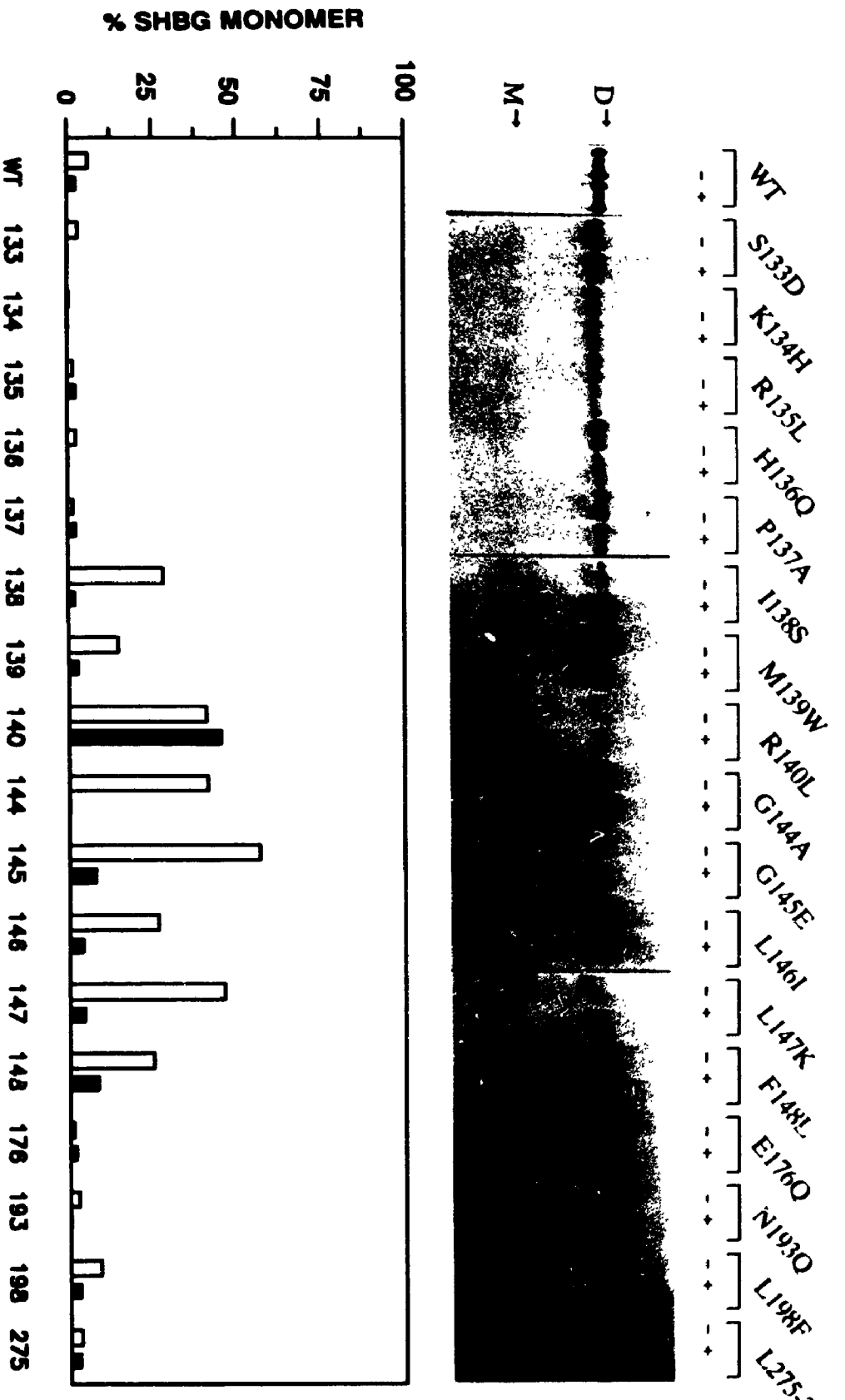
The SHBG mutants listed in Table 6.2 were also assessed for their ability to form stable dimers under different experimental conditions (Figures 6.4-6.9). The wild-type and mutant SHBGs were tested initially for dimer formation in the presence or absence of androgen (Figure 6.4). When steroid was removed from the samples, mutants containing amino acid substitutions at residues 138-148 were characterized by the presence of an immunoreactive form of SHBG that migrates with an R_f=0.50 (relative to the bromophenol blue dye-front) during nondenaturing-PAGE and this most probably represents a monomeric form of SHBG. 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, and 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.

Table 6.3 Affinities of wild-type SHBG and the M139W SHBG mutant for steroid ligands relative to 5α-dihydrotestosterone (DHT). The relative binding affinities of wild-type SHBG and the M139W SHBG mutant 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. The increase in affinity of the M139W SHBG mutant for certain steroids relative to wild-type SHBG is indicated by both an *arrow* and the *value in parenthesis*.

COMPETITOR STEROID	WILD-TYPE SHBG	M139W SHBG*	
5α-Dihydrotestosterone	100 %	100 %	
Testosterone	12.0	13.0	
5αA-3ß,17ß-diol	40.0	33.0	
Δ5A-3ß,17ß-diol	6.7	16.7	↑ (2.5x)
17ß-Estradiol	3.0	4.0	
19-nor-Testosterone	0.4	2.0	↑ (5x)

* M139W SHBG binds [^{3}H]DHT with 5x less affinity than wild-type SHBG.

Figure 6.4 Densitometric analysis of western blots of wild-type and mutant SHBGs in the presence (+) or absence (-) of androgen. 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 nondenaturing-PAGE (10% resolving gel). 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.


% SHBG MONOMER
0
25
50
75
100
WT 133 134 135 136 137 138 139 140 144 145 146 147 148 176 193 198 275
D→
M→
WT
S133D
K134H
R135L
H136Q
P137A
I138S
M139W
R140L
G144A
G145E
L146I
L147K
F148L
E176Q
N193Q
L198F
L275-281I

156

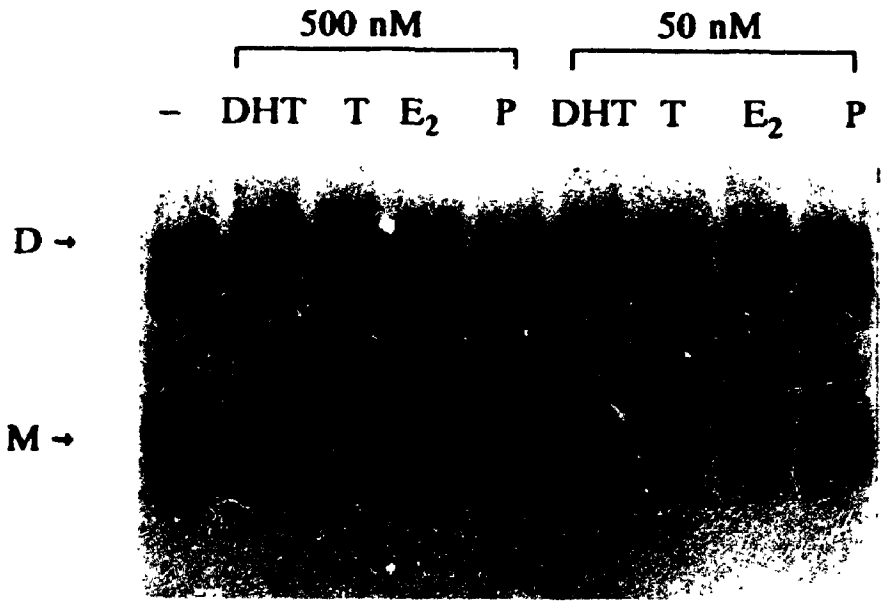
Densitometric analyses of the western blots provide a semi-quantitative assessment of the dimer instability of various SHBG mutants in the absence of steroid ligands (Figure 6.4). An UltrascanXL Enhanced Laser Densitometer (LKB) was used to scan a positive photographic image of the western blots, and after subtraction of background staining, the amount of immunoreactive monomeric SHBG was expressed as a percentage of the total immunoreactive SHBG in each lane. For this purpose, a rabbit polyclonal antiserum was selected that recognizes all 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 readdition of androgen largely restores the normal dimeric phenotype of all but one of the dimer-impaired mutants (Figure 6.4). 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 Ca^{2+} and DHT (Figure 6.7). Conversely, SHBG mutants with substitutions at residues 134-137, that show differences in their affinity for certain steroids (Table 6.2), dimerize normally irrespective of the presence or absence of androgen, as did wild-type SHBG and other mutants with substitutions outside the region Ile^{138} to Phe^{148} (Figure 6.4).

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, it was first DCC-treated to remove steroids from the binding site, and then exposed to various steroid ligands at two different concentrations. This demonstrated that DHT, T and E_2 were all effective at restoring dimer formation, while progesterone, which is not a ligand for SHBG, was not able to enhance dimer formation of this mutant even at a concentration of 500 nM (Figure 6.5).

6.3.4 Removal of Divalent Cations: Effects on Dimerization

Divalent cations were removed from culture medium samples by treatment with EDTA to ascertain whether this influences dimer formation of wild-type or mutant SHBGs, in the presence of 100 nM DHT. This resulted in the appearance of

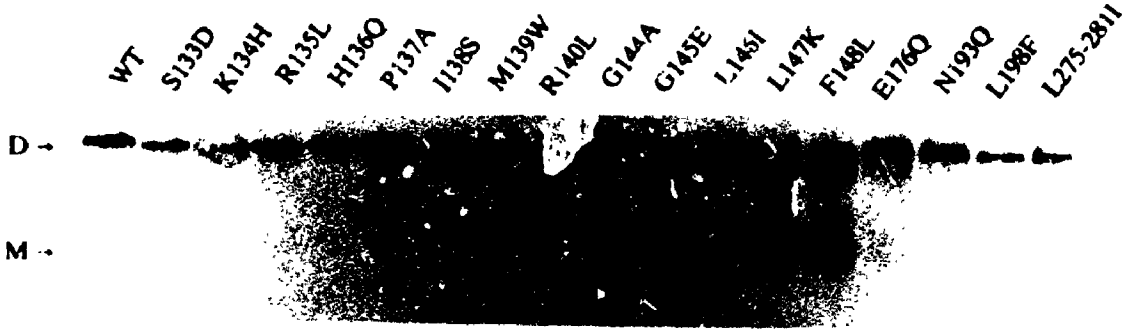
Figure 6.5 Western blot of the L147K SHBG mutant in the presence of different steroids. The DCC-treated L147K mutant was incubated alone (-) or in the presence of 5α-dihydrotestosterone (*DHT*), testosterone (*T*), 17ß-estradiol (E_2) and progesterone (*P*) at 500 nM and 50 nM concentrations, prior to nondenaturing-PAGE (10% resolving gel). The relative positions of normal dimeric (*D*) and monomeric (*M*) immunoreactive forms of SHBG are indicated (*arrows*).



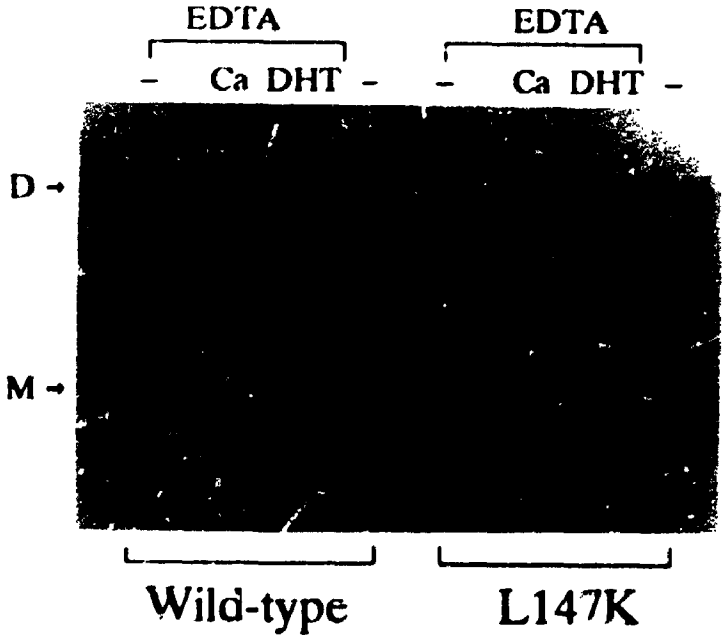
L147 K

Figure 6.6 Western blots of (A) wild-type and mutant SHBGs after treatment with EDTA, or (B) wild-type and L147K SHBGs after the removal and readdition of both 5α-dihydrotestosterone (*DHT*) and $CaCl_2$ (*Ca*). (A) Culture media containing wild-type and mutant SHBGs in the presence of 100 nM DHT were exposed to 5 mM EDTA for 15 min to remove divalent cations, prior to analysis by nondenaturing-PAGE (10% resolving gel). (B) 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 nondenaturing-PAGE (10% resolving gel). The relative positions of normal dimeric (*D*) and monomeric (*M*) immunoreactive forms of SHBG are indicated by *arrows* in both blots.

A


WT
S133D
K134H
R135L
H136Q
P137A
I138S
M139W
R140L
G144A
G145E
L145I
L147K
F148L
E176Q
N193Q
L198F
L275-281I
D
M

B


EDTA
EDTA
– Ca DHT –
– Ca DHT –
D
M
Wild-type
L147K

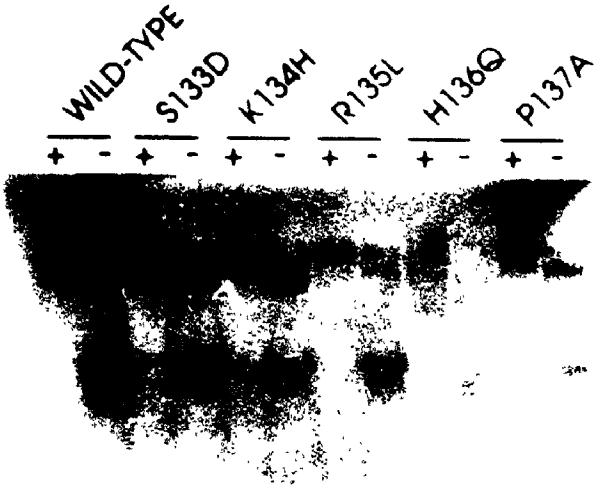
monomers in those mutants with amino acid substitutions at positions 140-148 (Figure 6.6A), which include the same mutants that exhibit a reduced ability to dimerize in the absence of steroid ligands (Figure 6.4). As in the case of wild-type SHBG, substitutions elsewhere in the protein had essentially no effect on dimerization in the absence of divalent cations (Figure 6.6A).

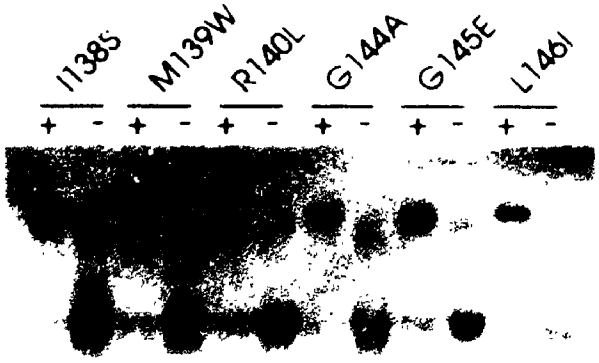
6.3.5 Removal of Steroids and Divalent Cations: Effects on Dimerization

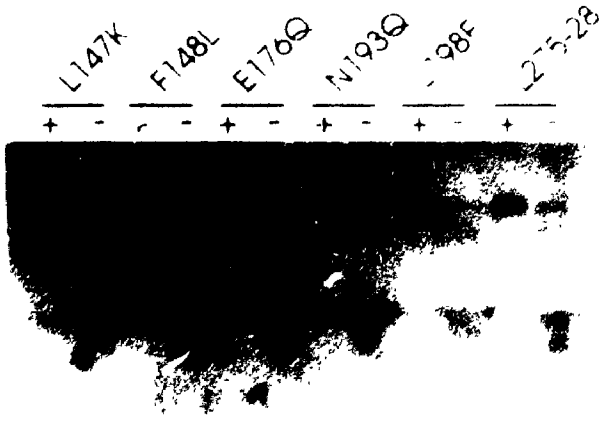
Since removal of steroid ligands or divalent cations destabilizes only those mutants containing amino acid substitutions at residues 140-148, both steroids and divalent cations were removed from culture media containing wild-type or L147K SHBG. This was done to determine whether the absence of both components could further exacerbate dimer instability. As expected, the L147K SHBG mutant completely lacked a normal dimeric phenotype under these conditions, but readdition of either DHT or Ca^{2+} regenerates dimer formation of this mutant (Figure 6.6B). It should be noted that the dimerization of wild-type SHBG was also compromised to a limited extent in the absence of both steroid and Ca^{2+}, and again readdition of either DHT or Ca^{2+} can restore dimer formation. This result was also important because it demonstrated that the diffuse band, with a slightly greater mobility than the normal SHBG dimer, disappeared after readdition of Ca^{2+} (Figure 6.6B), and supports our assumption that it represents a dimer with altered conformation, as opposed to being a degradation product.

All samples were then treated to remove both steroid and Ca^{2+} and it was found that SHBG mutants 138-148 completely lacked a normal dimeric phenotype while the remaining samples displayed a pattern of reduced dimer formation that is similar to wild-type SHBG (Figure 6.7). Again, the dimer with an altered conformation is apparent in most samples after the removal of both steroid and Ca^{2+}, however this dimeric form is not present after the readdition of both components (Figure 6.7).

Figure 6.7 Western blot of wild-type and mutant SHBGs in the presence (+) or absence (-) of both divalent cations and androgen. Culture media containing wild-type or mutants 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 and 10 mM $CaCl_2$ for 15 min at 37°C, followed by 45 min at room temperature before nondenaturing-PAGE (10% resolving gel).


WILD-TYPE
S133D
K134H
R135L
H136Q
P137A
+ - + - + - + - + - + -


I138S
M139W
R140L
G144A
G145E
L146I
+ - + - + - + - + - + -


L147K
F148L
E176Q
N193Q
+ - + - + - + - + -

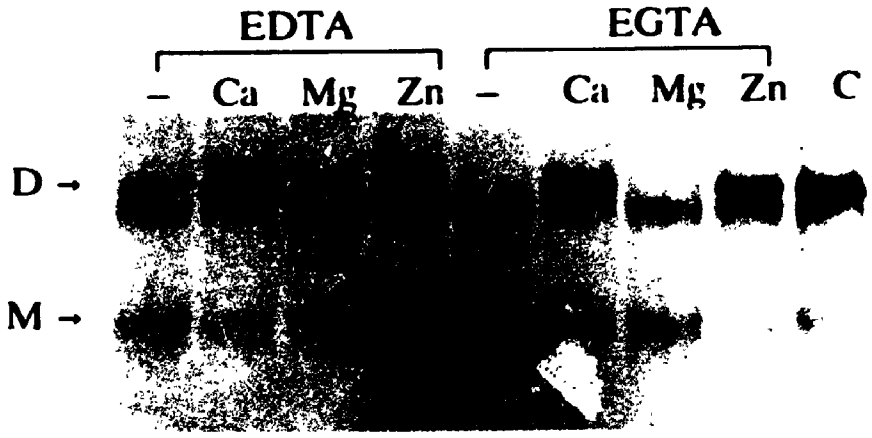
6.3.6 Presence of Different Divalent Cations: Effects on Dimerization

Although Ca^{2+} is able to restore SHBG dimer formation, Mg^{2+} is also present in the culture medium, and it was important to determine whether other divalent cations could produce the same effects as Ca^{2+}. To illustrate this, wild-type and L147K SHBG were treated separately with EDTA or EGTA in the presence of 100 nM DHT, and various divalent cations were then added back individually. After treatment with EDTA, the L147K SHBG mutant did not completely dissociate into monomer, and Ca^{2+} or Zn^{2+}, and to a limited extent, Mg^{2+}, were capable of regenerating dimer formation (Figure 6.8). By comparison, EGTA (a specific chelator of Ca^{2+}) is much more effective than EDTA at disrupting the L147K SHBG dimer, and in this case only Ca^{2+} and Zn^{2+} restored its normal dimeric state (Figure 6.8). These data suggest that Mg^{2+} does not bind to SHBG, and therefore the limited ability of Mg^{2+} to restore dimer formation in the EDTA-treated samples is probably due to displacement of bound Ca^{2+} from EDTA by the readdition of a 3-fold molar excess of Mg^{2+} over unoccupied EDTA sites. The normal dimeric phenotype of wild-type SHBG remained unaffected by any of the treatments, illustrating the specificity of these observations and confirming the stabilizing effect of both steroid and an intact dimerization domain.

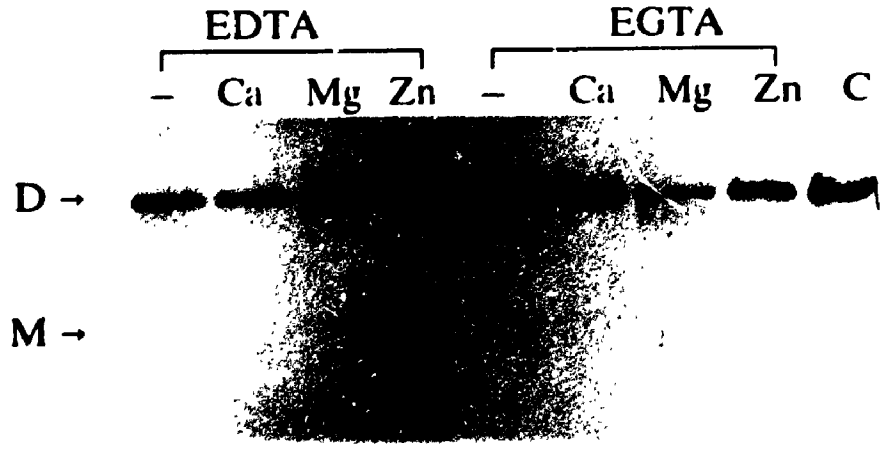
6.3.7 Immunochemical and Steroid-binding Properties of GST/SHBG Fusion Proteins

The data in Chapter 5 support the notion that the amino acids required for high-affinity steroid binding are located within the *N*-terminal 205 amino acids of human SHBG. However, it cannot be absolutely ruled out that residues in the *C*-terminal portion of human SHBG may contribute directly to the steroid-binding domain or may be required for the proper folding of the molecule to form a steroid-binding site. Unfortunately, SHBG mutants lacking either disulphide bridge, or a deletion mutant truncated at the *C*-terminus were not secreted from CHO cells, therefore, it was thought that the expression of truncated SHBG molecules lacking *C*-terminal regions, in *E. coli* might help resolve this issue. Equivalent amounts of each purified fusion protein, as determined by densitometry of proteins in

Figure 6.8 Western blot of wild-type and L147K SHBG 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*), 10 mM $ZnCl_2$ (*Zn*), prior to analysis by nondenaturing-PAGE (10% resolving gel). 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 by *arrows*.



L147K



Wild-type

Coomassie-stained gels, were examined by an IRMA and a steroid-binding capacity assay. There was a good correspondence between the steroid-binding capacity and the immunochemically-determined concentration of fusion proteins that contain at least 194 *N*-terminal amino acids of SHBG (Table 6.4). Removal of an additional 17 *C*-terminal residues to yield SHBG(1-177) resulted in the loss of Cys^{188}, which normally forms a disulphide bridge with Cys^{164} (Walsh *et al.*, 1986) and this caused a 7-fold discrepancy between its steroid-binding capacity and concentration measured by IRMA (Table 6.4). This relative decrease in immunoreactivity is probably due to the disruption of the conformation-dependent epitope recognized by the monoclonal antibody used in the IRMA (Hammond and Robinson, 1984; Hammond *et al.*, 1985). Furthermore, the SHBG(1-177) truncation mutant contained a lower steroid-binding capacity compared to the other fusion proteins, which suggests a loss of affinity due to an altered steroid-binding site (Table 6.4).

6.4 Discussion

Amino acid substitutions in a region encompassing Met^{139} of human SHBG induce marked changes in its steroid-binding and dimerization properties. The changes introduced in the human SHBG sequence were essentially confined to single amino acid substitutions, and this resulted in the secretion of all but three mutants. This experimental approach differs from that in a recent study in which radical alterations were made throughout the entire sequence of rat ABP, and which failed to locate any particular region that might represent the steroid-binding site (Joseph and Lawrence, 1993). Our rationale for choosing particular substitutions was based on a phylogenetic comparison of the primary structures of other sex steroid-binding proteins and species-specific differences in their steroid-binding affinities (Westphal, 1986). Almost all the substitutions introduced did not disrupt production and/or secretion of the recombinant proteins, and this permitted a detailed analysis of the steroid-binding properties of SHBG mutant proteins. Although, we cannot be certain that these mutations did not disrupt the protein structure, recombinant proteins that are misfolded during synthesis in mammalian cells are often poorly secreted and/or undergo rapid degradation within host cells

Table 6.4 Immunochemical and steroid-binding measurements of GST/SHBG fusion proteins. The GST/SHBG fusion proteins expressed in *E. coli* are named according to the region of the SHBG polypeptide (*residues in parentheses*) contained within their coding sequences. Equivalent amounts of fusion proteins were analyzed by an immunoradiometric assay (IRMA) specific for human SHBG and a steroid-binding capacity assay (BCA). The position of conserved cysteine residues participating in two intramolecular disulphide linkages are shown as *vertical lines* and labeled in SHBG(1-373).

Regions of SHBG expressed as GST fusion proteins		BCA	IRMA
NAME	DIAGRAM	(pmol/mL)	
SHBG(1-373)		20.0	26.0
SHBG(1-205)		16.9	26.0
SHBG(1-194)		26.8	23.7
SHBG(1-177)		9.4	1.4

(Rose and Doms, 1988). Since most of the SHBG mutants are secreted in amounts similar to wild-type SHBG, it is unlikely that their overall structures are substantially altered. Furthermore, this type of approach has been successfully applied in a similar study of the estrogen receptor, which established that it is was possible to discriminate between amino acids that influence either steroid binding or dimerization (Fawell *et al.*, 1990). More importantly, the retention of at least one of these functions further suggests that the changes introduced into the estrogen receptor, or human SHBG in our study, do not significantly alter the protein structure.

As demonstrated in Chapter 5, His^{136} in human SHBG is part of the epitope recognized by the monoclonal antibody used in the IRMA. In addition, mutations at Pro^{137}, Ile^{138}, Arg^{140}, and Leu^{147} also resulted in reduced immunoreactivity. It is not likely that the epitope conformation was altered due to a radical change in protein structure since these mutants were secreted and maintained either normal steroid-binding or dimerization ability. In addition, amino acid substitutions introduced at adjacent residues had no effect on measurements obtained by the IRMA. Therefore, residues 136-138 which reside in a region of SHBG that is poorly conserved between species, are probably the antigenic determinants of this epitope, responsible for the specific recognition of human SHBG by the monoclonal antibody. However, Arg^{140} and Leu^{147}, which are phylogenetically conserved, may not be antigenic *per se*, but probably make contact with the antigen-binding site on the antibody and substitution of these residues may hinder this interaction. It is also interesting that the monoclonal antibody recognizes a region that is involved in steroid-binding but does not itself interfere with this process (Hammond *et al.*, 1985).

It is well established that substitutions of Met^{139} in human SHBG almost invariably cause marked reductions in affinity for androgen (see Chapter 5) (Sui *et al.*, 1992). Studies described in this chapter reveal a phylogenetically poorly conserved region immediately *N*-terminal to Met^{139}, where substitutions result in

subtle but consistent alterations in steroid-binding specificity. Most notable of these are the abnormal affinities for E_2 that result from altering residues 134-138 in human SHBG. This suggests that the unique nonplanar configuration of E_2 (Table 6.1) results in the phenolic A ring being spatially closer to this series of residues that could be located 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 functional group (3ß-OH) at C3 that is also oriented above the plane of the molecule (Table 6.1) 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. A recent study of mutations in the analogous region of rat ABP also resulted in mutants with altered affinities for E_2 (Danzo and Joseph, 1994), but no other ligands were tested and this precludes any conclusions about how the steroid ligand might contact specific amino acids.

Further analyses of the M139W mutant has demonstrated that it not only binds DHT with 5-fold less affinity than wild-type SHBG but that its affinities for T, 5αA and E_2 are also similarly reduced (Table 6.2). By contrast, M139W SHBG binds 19norT with the same affinity as wild-type SHBG. It is therefore possible that the lack of a methyl group at C19 (Table 6.1) 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 (Table 6.1) and this could also explain the specificity of this effect. 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 A/B ring plane such that the binding of this steroid ligand by M139W SHBG is not hindered to the same degree as the other C19 steroids; DHT, T, and 5αA.

The S42L SHBG mutant did not exhibit any measurable steroid-binding affinity but was secreted in abundance and retained IRMA activity. This is the first

identification of an amino acid that could influence steroid binding, and which is situated further *N*-terminal to the region neighbouring Met^{139}. The specificity of this effect is illustrated by the fact that a mutation at Lys^{39} had no effect on the steroid-binding activity of SHBG.

It is probable that residues 134-139 normally make contact with the steroid ligand, and that substitutions introduced at these positions may perturb the SHBG steroid-binding site and allow it to either 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. This is consistent with the photoaffinity-labeling data that suggests Met^{139} interacts with the B ring of steroid ligands (Grenot *et al.*, 1988; Grenot *et al.*, 1992). The steroid D ring containing a 17ß-OH group is absolutely required for a high affinity interaction with the SHBG steroid-binding site, and it may be that Ser^{42} interacts with this region of the steroid since its substitution to a leucine completely abolishes detectable binding of DHT. This concept of the steroid-binding site contrasts with the affinity-labeling of Lys^{134} using a 17ß-bromoacetoxy derivative of DHT (Namkung *et al.*, 1990), but the conclusion that Lys^{134} interacts with the D ring of this affinity label is debatable because the 17ß-bromoacetoxy group is extremely reactive and bulky and may not accurately define which amino acid is closest to the steroid. This may explain why it has also been reported to interact with His^{235} (Khan and Rosner, 1990). Although, our assumptions are speculative in the absence of tertiary structural data about the SHBG steroid-binding site, once this information is obtained it will be important to incorporate these data from our studies into this model with respect to the interaction of specific amino acids with different structural features on the steroid ligand.

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 (Ile^{138} to Phe^{148}) clearly impair its ability to dimerize. In

addition, 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, together with the fact that these residues partially overlap with a region that influences steroid-binding activity, are interesting in view of reports that the steroid-binding and dimerization domains of the estrogen receptor overlap (Fawell *et al.,* 1990; White *et al.,* 1991), and that dimerization of the estrogen and androgen receptors is ligand-inducible (Kumar and Chambon, 1988; Wong *et al.,* 1993). Surprisingly, alterations immediately *C*-terminal to Met^{139} 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, and it is likely that loss of a particular local conformation and/or a hydrophobic residue may alter the nature of a hydrophobic interface that is essential for efficient subunit association.

In rabbit SHBG, residues 141 (leucine) and 143 (valine) differ from the isoleucine and leucine residues found in these respective positions in SHBG molecules from other species (Figure 1.3), and it was therefore assumed that they may not contribute to steroid-binding. On the other hand, Ala^{142} is invariant across species and its alteration to a charged residue (glutamic acid) resulted in a lack of secretion that 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 (Gly→Glu) and 146 (Leu→Lys) with more hydrophilic residues, and which also resulted in a lack of secretion. This suggests that these changes severely disrupted the structure of the molecule, especially as more conservative substitutions at these positions (G144A and L146I) resulted in the secretion of SHBG mutants with normal steroid-binding properties. However, these latter mutants were defective in their ability to dimerize in the absence of steroid, which implies that Gly^{144} and Leu^{146} may participate in the formation of a hydrophobic domain that is required for strong subunit association. It is also remarkable that these two substitutions

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 bound steroid and dimerized appropriately, and this suggests that the region of alternating leucines within the *C*-terminal portion of the molecule from Leu^{267} to Leu^{281} may not participate in either steroid binding or dimerization (Figure 1.3). It also further illustrates that the region identified as participating in dimerization in our studies is sensitive to even minor changes in the size (G144A) or spatial isomerization (L146I) of amino acid side chains.

Interestingly, 19 of the 24 residues at positions 139-162 in human SHBG are hydrophobic and well conserved with SHBG-related proteins from other species (Figure 1.3). If this region of human SHBG adopted an α-helical structure, then hydrophobic residues Met^{139}, Leu^{143}, Leu^{146}, Ala^{150}, Leu^{153}, Leu^{157}, and Ala^{161} would reside on the same face of the helix at every fourth then third position as required for the consensus sequence for a coiled-coil dimerization motif, $(abcdefg)_n$, where residues *a* and *d* are hydrophobic, and *e* and *g* are charged (O'Shea *et al.*, 1991). This would create a central hydrophobic core on one side of the helix which might permit interaction with hydrophobic residues on the other subunit. Furthermore, 3 of the 5 polar residues in this region, Arg^{140}, Arg^{154}, and Asp^{162} would reside on the same face as the core of hydrophobic residues away from the centre of the helix in positions *e* and *g*, that might permit ionic bonding with suitable residues on the other subunit. Again, in the absence of tertiary structure data this observation is speculative, but coupled with data in this chapter this region may adopt this structure and serve as a dimerization domain for SHBG.

Partially-purified SHBG rapidly and irreversibly loses steroid-binding activity, but this can be delayed if androgen and Ca^{2+} are present in the storage buffer (Rosner *et al.*, 1974). It is therefore interesting that removal of divalent cations from the culture medium destabilizes the dimerization of exactly the same 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

Ca^{2+} or androgen independently can promote dimer formation, and therefore their modes and sites of action in this regard are probably not associated. This is further supported by the fact that dimer formation of wild-type SHBG is only compromised in the absence of both divalent cation and steroid, and illustrates the greater efficiency of the intact dimerization domain in wild-type SHBG, as compared to the compromised state of the dimerization domain of L147K and other mutants with substitutions in this region.

It is not certain that Ca^{2+} is required to stabilize SHBG in biological fluids, but the results presented here demonstrate a degree of specificity in the way SHBG associates with cations because Mg^{2+} is clearly not involved in this process. Although it has been shown that there are four metal-binding sites per human and rabbit SHBG dimer (Ross *et al.*, 1985), 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). Furthermore, 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). Human SHBG exhibits sequence identity with protein S (Joseph and Baker, 1992); the Ca^{2+}-binding properties of which are an essential facet of its function as a cofactor in the blood coagulation pathway (Dahlbäck *et al.*, 1990). Zinc is an essential metal that is required for the development of the seminiferous tubules and spermatogenesis (Reed and Stitch, 1973; Maynard, 1965), and it may be possible that ABP not only transports androgen but also functions as a Zn^{2+}-transport protein in this compartment. This is not an unreasonable suggestion because transferrin produced by Sertoli cells has been demonstrated to deliver iron to the germ cell compartment *via* a receptor-mediated process (Petrie and Morales, 1992), and ceruloplasmin, which is also secreted by the Sertoli cells (Skinner and Griswold, 1983), is thought to deliver copper in a similar manner. Furthermore, an earlier study described the presence of a Zn^{2+}-binding protein that also bound androgens, in the cytosolic fraction of human prostate tissue homogenates and in seminal fluid (Reed and Stitch, 1973).

The correct tertiary structure of SHBG probably requires the formation of two intramolecular disulphide linkages, and this may explain the lack of secretion observed for SHBG mutants lacking either pair of cysteines that form these bridges. It is therefore likely that these bridges are necessary for appropriate folding of the molecule, and our immediate assumption was that one or both of them are required to ensure correct formation of either the dimerization and/or steroid-binding domains. It is known that the disulphide bridges do not link the subunits and because the quaternary structure of SHBG can apparently be disrupted and then regenerated by the readdition of steroid ligands, and Ca^{2+} or Zn^{2+}, it would appear that each SHBG subunit probably folds independently prior to dimerization. Whether dimerization occurs intracellularly during post-translational processing or during secretion is not known, but given the extremely low intracellular concentrations of Ca^{2+}, it likely associates with the protein extracellularly after dimerization has occurred in order to stabilize it.

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, 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 considered necessary to release the SHBG portions of these fusion proteins by thrombin digestion in order to demonstrate that the requirements for high-affinity binding of specific ligands are located within the *N*-terminal half of SHBG. These data confirm the *N*-terminal location of the steroid-binding domain suggested by our analyses of human SHBG/rat ABP chimeras (Chapter 5) and a series of SHBG mutants with amino acid substitutions in the region of Met^{139}. In addition, our results argue against the validity of the affinity-labeling of residues in the *C*-terminal portion of human SHBG (Hammond *et al.*, 1987; Petra *et al.*, 1988), including His^{235} (Khan and Rosner, 1990), which were thought to interact with steroid.

Many of the SHBG deletion mutants produced in *E. coli* bind steroids with high affinity. These findings contradict the notion that the entire molecule is necessary for the formation of the steroid-binding site (Joseph and Lawrence, 1993), and suggest that SHBG may have defined functional domains. This is an important finding because *Shbg* 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 peptide required for secretion (Hammond *et al.*, 1989; Joseph *et al.*, 1991b). The proteins encoded by these alternatively-spliced transcripts have not been identified, but the fact that SHBG deletion mutants bind steroids suggest that intracellular truncated forms of SHBG 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. 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 and 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.*, 1984b; Hryb *et al.*, 1989; Porto *et al.*, 1992b).

In conclusion, these data presented here are consistent with the hypothesis that structural elements important for high affinity steroid binding reside within the *N*-terminal half of SHBG. These data also suggest that amino acids at positions 134-139 interact with the A/B ring structures of steroid ligands while residues in a more *N*-terminal location, including Ser^{42}, may interact with another region of the steroid

ligand. The region cont: ning residues 134-139 partially overlaps with a series of predominantly hydrophobic : esidues (138-148) that appear to constitute an important component of the dimerization interface between SHBG monomers, and steroid ligands probably reside in close proximity to this interface. Divalent cations have also been demonstrated to be involved in stabilizing dimer formation, and the data presented here provide direct evidence that Ca^{2+} may be responsible for this. Given that several other steroid-binding proteins form homodimers in the presence of steroid, examples of which include uteroglobin (Miele *et al.*, 1987) and the estrogen and androgen receptors (Fawell *et al.*, 1990; White *et al.*, 1991; Kumar and Chambon, 1988; Wong *et al.*, 1993), further structural analysis of SHBG may provide insight into how these proteins associate as homodimers and bind steroid ligands. The *E. coli* expression of SHBG deletion mutants may be utilized to facilitate further delineation of the SHBG steroid-binding and dimerization domains, and could be useful for more definitive structural studies of these domains using nuclear magnetic resonance or X-ray diffraction techniques.

CHAPTER 7

CONCLUSIONS

7.1 Conclusions

The successful expression of recombinant human SHBG in mammalian cell lines provided a direct means of addressing many unresolved issues concerning SHBG biosynthesis and function. Analyses of recombinant SHBG demonstrated that the subunit size heterogeneity associated with natural SHBG is not due to proteolytic modification of the SHBG polypeptide in the blood or seminiferous tubule fluid, and can be accounted for by differences in glycosylation. Furthermore, our results provided evidence that tissue-specific differences in SHBG glycosylation occur *in vivo*.

The ability to produce recombinant SHBG together with the use of site-directed mutagenesis has permitted a more detailed analysis of the utilization of glycosylation sites and their impact on the structure and function of SHBG. In this way, I have been able to demonstrate that the subunit size heterogeneity associated with SHBG is due to the differential utilization of the two *N*-glycosylation consensus sites on each subunit, and that subunits comprising a given dimer are glycosylated and processed in the same way. The oligosaccharides associated with SHBG do not participate in steroid binding and are not essential for the folding process of the protein, but the lack of *N*-linked carbohydrates does affect SHBG production and/or secretion from cells.

The analysis of human SHBG/rat ABP chimeras has demonstrated that the amino acids responsible for the high affinity steroid-binding properties of human SHBG are located within the first 205 residues of these proteins. Furthermore, human SHBG mutants containing specific amino acid substitutions in the *N*-terminal half of the protein displayed alterations in steroid-binding affinity for specific ligands with unique structural properties. Amino acids at positions 134-139 interact with the A/B ring structures of steroid ligands, while residues in a more *N*-terminal location, including Ser^{42}, also contact steroid ligands. Analyses of human SHBG truncation mutants expressed in *E. coli* confirmed the *N*-terminal location of the steroid-binding site, and suggested that SHBG may have defined functional domains.

The *C*-terminal half of SHBG may provide structural elements that stabilize the steroid-binding site but it may also provide some other biological function, especially as it contains a conserved *N*-glycosylation site that may influence interactions with a plasma membrane receptor.

A dimerization domain was identified in human SHBG within a hydrophobic region of the molecule at residues 138-148, and it is likely that a particular local conformation adopted by the hydrophobic residues in this region contribute to the dimerization interface that is essential for efficient subunit association. Furthermore, steroid ligands probably reside in close proximity to this dimerization interface. The binding of steroids and divalent cations, specifically Ca^{2+} and Zn^{2+}, are required to maintain stable dimer formation, and their modes of action in this regard are probably independent. More definitive structural studies of the SHBG steroid-binding and dimerization domains will only be provided by physicochemical methods including nuclear magnetic resonance or X-ray diffraction techniques, and the expression of truncated SHBG molecules in *E. coli* may facilitate this process. Once this information is obtained, it will be important to incorporate data reported in this thesis into a model with respect to the impact of particular amino acids on the way specific steroid ligands gain entrance to, or reside within the steroid-binding site.

A library of human SHBG mutants with a wide range of unique structural and steroid-binding properties now exists, and these can be utilized for more detailed analyses of SHBG function. For example, the SHBG glycosylation mutants can be used to define the structural requirements for recognition by the SHBG receptor. Once the receptor is identified, it will be possible to determine the nature of the signalling event initiated by the binding of SHBG, and the effect of binding different SHBG mutants on receptor-mediated signalling can be studied. Furthermore, these mutants can be tested for their ability to affect the growth of human sex steroid-dependent cells in culture, and the ability to express human SHBG in nude mice provides a model to expand these studies *in vivo*.

REFERENCES

Ahrentsen, O.D., Jensen, H.K. and Johnsen, S.G. (1982) Sex-hormone-binding globulin deficiency. *Lancet* August: 377.

Ain, K.B., Mori, Y. and Refetoff, S. (1987) Reduced clearance rate of thyroxine-binding globulin (TBG) with increased sialylation: A mechanism for estrogen-induced elevation of serum TBG concentration. *J Clin Endocrinol Metab* **65**: 689-696.

Albelda, S.M. and Buck, C.A. (1990) Integrins and other cell adhesion molecules. *FASEB J* **4**: 2868-2880.

Anderson, D.C. (1974) Sex-hormone-binding globulin. *Clin Endocrinol (Oxf)* **3**: 69-96.

Auffray, C. and Rougeon, F. (1980) Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur J Biochem* **107**: 303-314.

Avvakumov, G.V., Matveentseva, I.V., Akhrem, L.V., Strel'chyonok, O.A. and Akhrem, A.A. (1983) Study of the carbohydrate moiety of human serum sex hormone-binding globulin. *Biochim Biophys Acta* **760**: 104-110.

Avvakumov, G.V., Zhuk, N.I. and Strel'chyonok, O.A. (1986) Subcellular distribution and selectivity of the protein-binding component of the recognition system for sex-hormone-binding protein-estradiol complex in human decidual endometrium. *Biochim Biophys Acta* **881**: 489-498.

Avvakumov, G.V., Zhuk, N.I. and Strel'chyonok, O.A. (1988) Biological function of the carbohydrate component of the human sex steroid-binding globulin. *Biochem USSR* **53**: 726-729.

Avvakumov, G.V., Warmels-Rodenhiser, S. and Hammond, G.L. (1993) Glycosylation of human corticosteroid binding globulin at Asn-238 is necessary for steroid binding. *J Biol Chem* **268**: 862-866.

Avvakumov, G.V. and Hammond, G.L. (1994) Glycosylation of human corticosteroid-binding globulin. Differential processing and significance of carbohydrate chains at individual sites. *Biochemistry* **33**: 5759-5765.

Babu, Y.S., Sack, J.S., Greenhough, T.J., Bugg, C.E., Means, A.R. and Cook, W.J. (1985) Three-dimensional structure of calmodulin. *Nature* **315**: 37-40.

Baker, M.E., French, F.S. and Joseph, D.R. (1987) Vitamin K-dependent protein S is similar to rat androgen binding protein. *Biochem J* **243**: 293-296.

Baker, S.J., Fearon, E.R., Nigro, J.M., et al. (1989) Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science* **244**: 217-221.

Bardin, C.W., Gunsalus, G.L., Musto, N.A., et al. (1988) Corticosteroid binding globulin, testosterone-estradiol binding globulin and androgen binding protein belong to protein families distinct from steroid receptors. *J Steroid Biochem* **30**: 131-139.

Bérubé, D., Seralini, G-E., Gagné, R. and Hammond, G.L. (1990) Localization of the human sex hormone-binding globulin gene (SHBG) to the short arm of chromosome 17 (17p12-p13). *Cytogenet Cell Genet* **54**: 65-67.

Birnboim, H.C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**: 1513-1523.

Bordin, S. and Petra, P.H. (1980) Immunocytochemical localization of the sex steroid-binding protein of plasma in tissues of the adult monkey *Macaca nemestrina*. *Proc Natl Acad Sci USA* **77**: 5678-5682.

Braunstein, G.D., Vaitukaitis, J.L. and Ross, G.T. (1972) The *in vivo* behavior of human chorionic gonadotropin after dissociation into subunits. *Endocrinology* **91**: 1030-1036.

Casali, E., Petra, P.H. and Ross, J.B.A. (1990) Fluorescence investigation of the sex steroid binding protein of rabbit serum: Steroid binding and subunit dissociation. *Biochemistry* **29**: 9334-9343.

Chaney, W.G., Howard, D.R., Pollard, J.W., Sallustio, S. and Stanley, P. (1986) High-frequency transfection of CHO cells using Polybrene. *Somat Cell Mol Genet* **12**: 237-244.

Chapman, R.S. and Ratcliffe, J.G. (1982) Covalent linkage of antisera to microparticulate cellulose using 1,1'-carbonyldiimidazole: A rapid, practical method with potential use in solid-phase immunoassay. *Clin Chim Acta* **118**: 129-134.

Cheng, C.Y., Musto, N.A., Gunsalus, G.L. and Bardin, C.W. (1983) Demonstration of heavy and light promotors of human testosterone-estradiol-binding globulin. *J Steroid Biochem* **19**: 1379-1389.

Cheng, C.Y., Gunsalus, G.L., Musto, N.A. and Bardin, C.W. (1984) The heterogeneity of rat androgen-binding protein in serum differs from that in testis and epididymis. *Endocrinology* **114**: 1386-1394.

Cheng, C.Y., Musto, N.A., Gunsalus, G.L. and Bardin, C.W. (1985a) The role of the carbohydrate moiety on the size heterogeneity and immunologic determinants of human testosterone-estradiol-binding globulin. *J Steroid Biochem* **22**: 127-134.

Cheng, C.Y., Musto, N.A., Gunsalus, G.L., Frick, J. and Bardin, C.W. (1985b) There are two forms of androgen binding protein in human testes. *J Biol Chem* **260**: 5631-5640.

Combarnous, Y. (1992) Molecular basis of the specificity of binding of glycoprotein hormones to their receptors. *Endocrine Rev* **13**: 670-691.

Concha, N.O., Head, J.F., Kaetzel, M.A., Dedman, J.R. and Seaton, B.A. (1993) Rat annexin V crystal structure: Ca^{2+}-induced conformational changes. *Science* **261**: 1321-1324.

Crave, J.C., Lejeune, H., Brebant, C., Baret, C. and Pugeat, M. (1994) Differential effects of insulin and insulin-like growth factor-1 on the production of plasma steroid binding globulins by human hepatoblastoma-derived (Hep G2) cells. *J Clin Endocrinol Metab* (In Press)

Cunningham, G.R., Tindall, D.J., Lobl, T.J., Campbell, J.A. and Means, A.R. (1981) Steroid structural requirements for high affinity binding to human sex steroid binding protein. *Steroids* **38**: 243-262.

Dahlbäck, B., Frohm, B. and Nelsestuen, G. (1990) High affinity interaction between C4b-binding protein and vitamin K-dependent protein S in the presence of calcium. *J Biol Chem* **265**: 16082-16087.

Danzo, B.J., Bell, B.W. and Black, J.H. (1989) Human testosterone-binding globulin is a dimer composed of two identical protomers that are differently glycosylated. *Endocrinology* **124**: 2809-2817.

Danzo, B.J., Parrott, J.A. and Skinner, M.K. (1991) Analysis of the steroid binding domain of rat androgen-binding protein. *Endocrinology* **129**: 690-696.

Danzo, B.J. and Black, J.H. (1990a) Structure of asparagine-linked oligosaccharides on human and rabbit testosterone-binding globulin. *Biol Reprod* **42**: 472-482.

Danzo, B.J. and Black, J.H. (1990b) Analysis of the oligosaccharides on rat androgen-binding protein using serial lectin chromatography. *Biol Reprod* **43**: 219-228.

Danzo, B.J. and Joseph, D.R. (1994) Structure-function relationships of rat androgen-binding protein/human sex-hormone binding globulin: The effect of mutagenesis on steroid-binding parameters. *Endocrinology* **135**: 157-167.

Duax, W.L. and Norton, D.A.(eds) *Atlas of Steroid Structure*, New York:Plenum Publishing, 1975.

Dunn, J.F., Nusula, B.C. and Rodbard, D. (1981) Transport of steroid hormones: Binding of 21 endogenous steroids to both testosterone-binding globulin in human plasma. *J Clin Endocrinol Metab* **53**: 58-68.

Egloff, M., Vendrely, E., Tardivel-Lacombe, J., Dadoune, J-P. and Degrelle, H. (1982) Etude immunohistochimique du testicule et de l'epididyme humains à l'aide d'un antisérum monospécifique dirigé contre la protéine plasmatique liant les hormones sexuelles. *C R Academy of Science* **295**: 107-111.

Engesser, L., Broekmans, A.W., Briet, E., Brommer, E.J.P. and Bertina, R.M. (1987) Hereditary protein S deficiency: Clinical manifestations. *Annals of Internal Medicine* **106**: 677-682.

Engvall, E., Earwicker, D., Day, A., Muir, D., Manthorpe, M. and Paulsson, M. (1992) Merosin promotes cell attachment and neurite outgrowth and is a component of the neurite-promoting factor of RN22 schwannoma cells. *Exp Cell Res* **198**: 115-123.

Esmon, C.T. (1987) The Regulation of Natural Anticoagulant Pathways. *Science* **235**: 1348-1352.

Fawell, S.E., Lees, J.A. and Parker, M.G. (1989) A proposed consensus steroid-binding sequence - a reply. *Mol Endocrinol* **3**: 1002-1005.

Fawell, S.E., Lees, J.A., White, R. and Parker, M.G. (1990) Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor. *Cell* **60**: 953-962.

Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**: 6-13.

Feldman, M., Lea, O.A., Petrusz, P., Tres, L.L., Kierszenbaum, A.L. and French, F.S. (1981) Androgen-binding protein. Purification from rat epididymis, characterization, and immunocytochemical localization. *J Biol Chem* **256**: 5170-5175.

Fotherby, K. (1988) Interactions of contraceptive steroids with binding proteins and the clinical implications. *Ann N Y Acad Sci* **538**: 313-320.

French, F.S. and Ritzen, E.M. (1973) A high-affinity androgen binding protein (ABP) in rat testis: Evidence for secretion into efferent duct fluid and absorption by epididymis. *Endocrinology* **93**: 88-95.

Furie, B. and Furie, B.C. (1988) The molecular basis of blood coagulation. *Cell* **53**: 505-518.

Galway, A.B., Hsueh, A.J.W., Keene, J.L., Yamoto, M., Fauser, B.C.J.M. and Boime, I. (1990) *In vitro* and *in vivo* bioactivity of recombinant human follicle-stimulating hormone and partially deglycosylated variants secreted by transfected eukaryotic cell lines. *Endocrinology* **127**: 93-100.

Gehlsen, K.R., Sriramarao, P., Furcht, L.T. and Skubitz, A.P.N. (1992) A synthetic peptide derived from the carboxy terminus of the laminin A chain represents a binding site for the $\alpha_1\beta_1$ integrin. *J Cell Biol* **117**: 449-459.

Gerard, A., Egloff, M., Gerard, H., et al. (1990) Internalization of human sex steroid-binding protein in the monkey epididymis. *J Mol Endocrinol* **5**: 239-251.

Gerard, A., En Nya, A., Egloff, M., Domingo, M., Degrelle, H. and Gerard, H. (1991) Endocytosis of human sex steroid-binding protein in monkey germ cells. *Ann N Y Acad Sci* **637**: 258-276.

Gerard, H., Gerard, A., En Nya, A., Felden, F. and Gueant, J.L. (1994) Spermatogenic cells do internalize sertoli androgen-binding protein: A transmission electron microscopy autoradiographic study in the rat. *Endocrinology* **134**: 1515-1527.

Gershagen, S., Henningsson, K. and Fernlund, P. (1987) Subunits of human sex hormone binding globulin. *J Biol Chem* **262**: 8430-8437.

Grenot, C., De Montard, A., Blachere, T., Mappus, E. and Cuilleron, C-Y. (1988) Identification d'un site de photomarquage de la protéine plasmatique de liaison de la testostérone et de l'oestradiol (SBP) par l'hydroxy-17β oxo-3 androstadiéne-4,6 tritié. *C R Academy of Science* **307**: 391-396.

Grenot, C., De Montard, A., Blachere, T., Rolland de Ravel, M., Mappus, E. and Cuilleron, C.Y. (1992) Characterization of Met-139 as the photolabeled amino acid residue in the steroid binding site of sex hormone binding globulin using Δ^6 derivatives of either testosterone or estradiol as unsubstituted photoaffinity labeling reagents. *Biochemistry* **31**: 7609-7621.

Griffin, P.R., Kumar, S., Shabanowitz, J., et al. (1989) The amino acid sequence of the sex steroid-binding protein of rabbit serum. *J Biol Chem* **264**: 19066-19075.

Gunsalus, G.L., Hershey, M.S., Musto, N.A. and Bardin, C.W. (1978) Immunoassay of androgen binding protein in blood: A new approach for study of the seminiferous tubule. *Science* **200**: 65-66.

Hagen, F.S., Arguelles, C., Sui, L-M., et al. (1992) Mammalian expression of the human sex steroid-binding protein of plasma (SBP or SHBG) and testis (ABP). *FEBS Lett* **299**: 23-27.

Hagenas, L., Ritzen, E.M., Ploen, L., Hansson, V., French, F.S. and Nayfeh, S.N. (1975) Sertoli cell origin of testicular androgen-binding protein (ABP). *Mol Cell Endocrinol* **2**: 339-350.

Hammond, G.L., Langley, M.S. and Robinson, P.A. (1985) A liquid-phase immunoradiometric assay (IRMA) for human sex hormone binding globulin (SHBG). *J Steroid Biochem* **23**: 451-460.

Hammond, G.L., Robinson, P.A., Sugino, H., Ward, D.N. and Finne, J. (1986) Physicochemical characteristics of human sex hormone binding globulin: Evidence for two identical subunits. *J Steroid Biochem* **24**: 815-824.

Hammond, G L., Underhill, D.A., Smith, C.L., et al. (1987) The cDNA-deduced primary structure of human sex hormone binding globulin and location of its steroid binding domain. *FEBS Lett* **215**: 100-104.

Hammond, G.L., Underhill, D.A., Rykse, H.M. and Smith, C.L. (1989) The human sex hormone binding globulin gene contains exons for androgen binding protein and two other testicular messenger RNAs. *Mol Endocrinol* **3**: 1869-1876.

Hammond, G.L. (1990) Molecular properties of corticosteroid binding globulin and the sex-steroid binding proteins. *Endocrine Rev* **11**: 65-79.

Hammond, G.L. (1993) Extracellular steroid binding proteins. In: *Steroid Hormone Action: Frontiers in Molecular Biology*, Edited by M. Parker) IRL Press at Oxford University, Oxford, England. pp. 1-25.

Hammond, G.L., Bocchinfuso, W.P., Orava, M., Smith, C.L., vandenEnde, A. and Waijjen, V. (1994) Serum distribution of two oral contraceptive progestins: 3-ketodesogestrel and gestodene. *Contraception* **50**: 301-318.

Hammond, G.L. and Lähteenmäki, P.L.A. (1983) A versatile method for the determination of serum cortisol binding globulin and sex hormone binding globulin binding capacities. *Clin Chim Acta* **132**: 101-110.

Hammond, G.L. and Robinson, P.A. (1984) Characterization of a monoclonal antibody to human sex-hormone binding globulin. *FEBS Lett* **168**: 307-312.

Hanahan, D. (1985) Techniques for transformation of *E. coli*. In: *DNA cloning Volume 1: A Practical Approach*, Edited by D.M. Glover) IRL Press, Oxford, England. pp. 109-135.

Hard, K., Mekking, A., Damm, J.B.L., et al. (1990) Isolation and structure determination of the intact sialylated N-linked carbohydrate chains of recombinant human follitropin expressed in Chinese hamster ovary cells. *Eur J Biochem* **193**: 263-271.

Hori, T., Takahashi, E. and Murata, M. (1988) Nature of distamycin A-inducible fragile sites. *Cancer Genet Cytogenet* **34**: 189-194.

Hossner, K.L. and Billiar, R.B. (1981) Plasma clearance and organ distribution of native and desialylated rat and human transcortin: Species specificity. *Endocrinology* **108**: 1780-1786.

Hryb, D.J., Khan, M.S. and Rosner, W. (1985) Testosterone-estradiol-binding globulin binds to human prostatic cell membranes. *Biochem Biophys Res Commun* **128**: 432-440.

Hryb, D.J., Khan, M.S., Romas, N.A. and Rosner, W. (1989) Solubilization and partial characterization of the sex hormone-binding globulin receptor from human prostate. *J Biol Chem* **264**: 5378-5383.

Hryb, D.J., Khan, M.S., Romas, N.A. and Rosner, W. (1990) The control of the interaction of sex hormone-binding globulin with its receptor by steroid hormones. *J Biol Chem* **265**: 6048-6054.

Hsu, A-F. and Troen, P. (1978) An androgen binding protein in the testicular cytosol of human testis. *J Clin Invest* **61**: 1611-1619.

Hynes, R.O. (1987) Integrins: A family of cell surface receptors. *Cell* **48**: 549-554.

Hynes, R.O. (1992) Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* **69**: 11-25.

Jaouen, G., Vessieres, A. and Butler, I.S. (1993) Bioorganometallic Chemistry: A future direction for transition metal organometallic chemistry? *Acc Chem Res* **26**: 361-369.

Joseph, D.R., Hall, S.H., Conti, M. and French, F.S. (1988) The gene structure of rat androgen-binding protein: Identification of potential regulatory deoxyribonucleic acid elements of a follicle-stimulating hormone-regulated protein. *Mol Endocrinol* **2**: 3-13.

Joseph, D.R., Sullivan, P.M., Wang, Y-M., et al. (1990) Characterization and expression of the complementary DNA encoding rat histidine decarboxylase. *Proc Natl Acad Sci USA* **87**: 733-737.

Joseph, D.R., Adamson, M.C. and Kozak, C.A. (1991a) Genetic mapping of the gene for androgen-binding protein/sex hormone-binding globulin to mouse chromosome 11. *Cytogenet Cell Genet* **56**: 122-124.

Joseph, D.R., Sullivan, P.M., Wang, Y-M., Millhorn, D.E. and Bayliss, D.M. (1991b) Complex structure and regulation of the ABP/SHBG gene. *J Steroid Biochem Molec Biol* **40**: 771-775.

Joseph, D.R., Lawrence, W. and Danzo, B.J. (1992) The role of asparagine-linked oligosaccharides in the subunit structure, steroid binding, and secretion of androgen-binding protein. *Mol Endocrinol* **6**: 1127-1134.

Joseph, D.R. and Baker, M.E. (1992) Sex hormone-binding globulin, androgen-binding protein, and vitamin K-dependent protein S are homologous to laminin A, merosin, and *Drosophila crumbs* protein. *FASEB J* **6**: 2477-2481.

Joseph, D.R. and Lawrence, W. (1993) Mutagenesis of essential functional residues of rat androgen-binding protein/sex hormone-binding globulin. *Mol Endocrinol* **7**: 488-496.

Keene, J.L., Matzuk, M.M. and Boime, I. (1989) Expression of recombinant human choriogonadotropin in Chinese hamster ovary glycosylation mutants. *Mol Endocrinol* **3**: 2011-2017.

Khan, M.S., Knowles, B.B., Aden, D.P. and Rosner, W. (1981) Secretion of testosterone-estradiol-binding globulin by a human hepatoma-derived cell line. *J Clin Endocrinol Metab* **53**: 448-449.

Khan, M.S., Ehrlich, P., Birken, S. and Rosner, W. (1985) Size isomers of testosterone-estradiol-binding globulin exist in the plasma of individual men and women. *Steroids* **45**: 463-472.

Khan, M.S., Hryb, D.J., Hashim, G.A., Romas, N.A. and Rosner, W. (1990) Delineation and synthesis of the membrane receptor-binding domain of sex hormone-binding globulin. *J Biol Chem* **265**: 18362-18365.

Khan, M.S. and Rosner, W. (1990) Histidine 235 of human sex hormone-binding globulin is the covalent site of attachment of the nucleophilic steroid derivative, 17ß-bromoacetoxydihydrotestosterone. *J Biol Chem* **265(15)**: 8431-8435.

Krupenko, N.I., Avvakumov, G.V. and Strel'chyonok, O.A. (1990) Binding of human sex hormone-b'nding globulin-androgen complexes to the placental syncytiotrophoblast membrane. *Biochem Biophys Res Commun* **171**: 1279-1283.

Krusius, T., Finne, J. and Rauvala, H. (1976) The structural basis of the different affinities of two types of acidic N-glycosidic glycopeptides for concanavalin A-sepharose. *FEBS Lett* **71**: 117-120.

Kumar, V. and Chambon, P. (1988) The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell* **55**: 145-156.

Larrea, F., Diaz, L., Carino, C., et al. (1993) Evidence that human placenta is a site of sex hormone-binding globulin gene expression. *J Steroid Biochem Molec Biol* **46**: 497-505.

Larriva-Sahd, J., Orozco, H., Hernandez-Pando, R., Oliart, R.M., Musto, N.A. and Larrea, F. (1991) Immunhistochemical demonstration of androgen-binding protein in the rat prostatic gland. *Biol Reprod* **45**: 417-423.

Lee, I.R., Dawson, S.A., Wetherall, J.D. and Hahnel, R. (1987) Sex hormone-binding globulin secretion by human hepato-carcinoma cells is increased by both estrogens and androgens. *J Clin Endocrinol Metab* **64**: 825-831.

Lehrman, M.A., Goldstein, J.L., Russell, D.W. and Brown, M.S. (1987) Duplication of seven exons in LDL receptor gene caused by Alu-Alu recombination in a subject with familial hypercholesterolemia. *Cell* **48**: 827-835.

Lobo, R.A. and Kletzky, O.A. (1983) Normalization of androgen and sex hormone-binding globulin levels after treatment of hyperprolactinemic. *J Clin Endocrinol Metab* **56**: 562-566.

Luckock, A. and Cavalli-Sforza, L.L. (1983) Detection of genetic variation with radioactive ligands. V. Genetic variants of testosterone-binding globulin in human serum. *Am J Hum Genet* **35**: 49-57.

Matzuk, M.M., Krieger, M., Corless, C.L. and Boime, I. (1987) Effects of preventing O-glycosylation on the secretion of human chorionic gonadotropin in Chinese hamster ovary cells. *Proc Natl Acad Sci USA* **84**: 6354-6358.

Maynard, E.A. (1965) Gold, Silver, Manganese and Other Minor Metals. In: *Drill's Pharmacology in Medicine*, Edited by J.R. DiPalma) McGraw-Hill Inc., New York. pp. 872-887.

McKinnon, R., Danielson, P, Brow, M.A.D., Bloom, F.E. and Sutcliffe, J.G. (1987) Expression of small cytoplasmic transcripts of the rat identifier element in vivo and in cultured cells. *Mol Cell Biol* **7**: 2148-2154.

Mercier-Bodard, C., Nivet, V. and Baulieu, E-E. (1991) Effects of hormones on SBP mRNA levels in human cancer cells. *J Steroid Biochem Molec Biol* **40**: 777-785.

Miele, L., Cordella-Miele, E. and Mukherjee, A.B. (1987) Uteroglobin: Structure, molecular biology, and new perspectives on its function as a phospholipase A_2 inhibitor. *Endocrine Rev* **8**: 474-490.

Mischke, W., Weise, H.C., Graesslin, D., Rusch, R. and Tamm, J. (1979) Isolation of highly purified sex hormone binding globulin (SHBG): Evidence for microheterogeneity. *Acta Endocrinologica (Copenhagen)* **90**: 737-742.

Mooradian, A.D., Morley, J.E. and Korenman, S.G. (1987) Biological actions of androgens. *Endocrine Rev* **8**: 1-28.

Nakhla, A.M., Khan, M.S. and Rosner, W. (1990) Biologically active steroids activate receptor-bound human sex hormone-binding globulin to cause LNCaP cells to accumulate adenosine 3',5'-monophosphate. *J Clin Endocrinol Metab* **71**: 398-404.

Nakhla, A.M., Khan, M.S., Romas, N.P. and Rosner, W. (1994) Estradiol causes the rapid accumulation of cAMP in human prostate. *Proc Natl Acad Sci USA* **91**: 5402-5405.

Namkung, P.C., Kumar, S., Walsh, K.A. and Petra, P.H. (1990) Identification of lysine 134 in the steroid-binding site of the sex steroid-binding protein of human plasma. *J Biol Chem* **265**: 18345-18350.

Narasimhan, S., Wilson, J.R., Martin, E. and Schachter, H. (1979) A structural basis for four distinct elution profiles on concanavalin A - Sepharose affinity chromatography of glycopeptides. *Can J Biochem* **57**: 83-96.

Nisker, J.A., Hammond, G.L., Davidson, B.J., et al. (1980) Serum sex hormone-binding globulin capacity and the percentage of free estradiol in postmenopausal women with and without endometrial cancer. *Am J Obstet & Gynecol* **138**: 637-641.

O'Shea, E.K., Klemm, J.D., Kim, P.S. and Alber, T. (1991) X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. *Science* **254**: 539-544.

Pardridge, W.M. (1981) Transport of protein-bound hormones into tissues *in vivo*. *Endocrine Rev* **2**: 103-123.

Pelliniemi, L.J., Dym, M., Gunsalus, G.L., Musto, N.A., Bardin, C.W. and Fawcett, D.W. (1981) Immunocytochemical localization of androgen-binding protein in the male rat reproductive tract. *Endocrinology* **108**: 925-931.

Pelliniemi, L.J., Dym, M., Chemes, H.E., Gunsalus, G.L., Musto, N.A. and Bardin, C.W. (1986) Structural evidence for the intracellular presence of androgen-binding protein. In: *Binding Proteins of Steroid Hormones*, Volume 149. (Edited by M.G. Forest and M. Pugeat) John Libbey & Company, Ltd., London, England. pp. 537-544.

Petra, P.H., Kumar, S., Hayes, R., Ericsson, L.H. and Titani, K. (1986) Molecular organization of the sex steroid-binding protein (SBP) of human plasma. *J Steroid Biochem* **24**: 45-49.

Petra, P.H., Que, B.G., Namkung, P.C., et al. (1988) Affinity labeling, molecular cloning, and comparative amino acid sequence analyses of sex steroid-binding protein of plasma. *Ann N Y Acad Sci* **538**: 10-24.

Petra, P.H. (1991) The plasma sex steroid binding protein (SBP or SHBG). A critical review of recent developments on the structure, molecular biology and function. *J Steroid Biochem Molec Biol* **40**: 735-753.

Petra, P.H., Griffen, P.R., Yates III, J.R., Moore, K. and Zhang, W. (1992) Complete enzymatic deglycosylation of native sex steroid-binding protein (SBP or SHBG) of human and rabbit plasma: Effect on the steroid-binding activity. *Protein Sci* **1**: 902-909.

Petrie, R.G.,Jr. and Morales, C.R. (1992) Receptor-mediated endocytosis of testicular transferrin by germinal cells of the rat testis. *Cell Tissue Res* **267**: 45-55.

Picado-Leonard, J. and Miller, W.L. (1988) Homologous sequences in steroidogenic enzymes, steroid receptors and a second binding protein suggest a consensus steroid-binding sequence. *Mol Endocrinol* **2**: 1145-1150.

Plymate, S.R., Matej, L.A., Jones, R.E and Friedl, K.E. (1988) Inhibition of sex hormone-binding globulin production in the human hepatoma (Hep G2) cell line by insulin and prolactin. *J Clin Endocrinol Metab* **67**: 460-464.

Plymate, S.R., Loop, S.M., Hoop, R.C., et al. (1991) Effects of sex hormone binding globulin (SHBG) on human prostatic carcinoma. *J Steroid Biochem Molec Biol* **40**: 833-839.

Poller, L. (1978) Oral contraceptives, blood clotting and thrombosis. *Br Med Bull* **34**: 151-156.

Porto, C.S., Gunsalus, G.L., Bardin, C.W., Phillips, D.M. and Musto, N.A. (1991) Receptor-mediated endocytosis of an extracellular steroid-binding protein (TeBG) in MCF-7 human breast cancer cells. *Endocrinology* **129**: 436-445.

Porto, C.S., Abreu, L.C., Gunsalus, G.L. and Bardin, C.W. (1992a) Binding of sex-hormone-binding globulin (SHBG) to testicular membranes and solubilized receptors. *Mol Cell Endocrinol* **89**: 33-38.

Porto, C.S., Musto, N.A., Bardin, C.W. and Gunsalus, G.L. (1992b) Binding of an extracellular steroid-binding globulin to membranes and soluble receptors from human breast cancer cells (MCF-7 cells). *Endocrinology* **130**: 2931-2936.

Pugeat, M., Crave, J.C., Elmidani, M., et al. (1991) Pathophysiology of sex hormone binding globulin (SHBG): Relation to insulin. *J Steroid Biochem Molec Biol* **40**: 841-849.

Pugeat, M.M., Dunn, J.F. and Nisula, B.C. (1981) Transport of steroid hormones: Interaction of 70 drugs with testosterone-binding globulin and corticosteroid-binding globulin in human plasma. *J Clin Endocrinol Metab* **53**: 69-75.

Rademacher, T.W., Parekh, R.B. and Dwek, R.A (1988) Glycobiology. *Annu Rev Biochem* **57**: 785-838.

Raggatt, L.E., Blok, R.B., Hamblin, P.S. and Barlow, J.W. (1992) Effects of thyroid hormone on sex hormone-binding globulin gene expression in human cells. *J Clin Endocrinol Metab* **75**: 116-120.

Rave, N., Crkvenjakov, R. and Boedtker, H. (1979) Identification of procollagen mRNAs tranferred to diazobenzyloxymethyl paper from formaldehyde agarose gels. *Nucleic Acids Res* **6**: 3559-3567.

Reed, M.J. and Stitch, S.R. (1973) The uptake of testosterone and zinc *in vitro* by the human benign hypertrophic prostate. *J Endocrinol* **58**: 405-419.

Reventos, J., Hammond, G.L., Crozat, A., et al. (1988) Hormonal regulation of rat androgen-binding protein (ABP) messenger ribonucleic acid and homology of human testosterone-estradiol-binding globulin and ABP complementary deoxyribonucleic acids. *Mol Endocrinol* **2**: 125-132.

Reventos, J., Sullivan, P.M., Joseph, D.R. and Gordon, J.W. (1993) Tissue-specific expression of the rat androgen-binding protein/sex hormone-binding globulin gene in transgenic mice. *Mol Cell Endocrinol* **96**: 69-73.

Rose, J.K. and Doms, R.W. (1988) Regulation of protein export from the endoplasmic reticulum. *Ann Rev Cell Biol* **4**: 257-288.

Rosner, W., Toppel, S. and Smith, R.N. (1974) Testosterone-estradiol-binding globulin of human plasma: Denaturation and protection. *Biochim Biophys Acta* **351**: 92-98.

Rosner, W. (1990) The functions of corticosteroid-binding globulin and sex hormone-binding globulin: Recent advances. *Endocrine Rev* **11**: 80-91.

Rosner, W. (1992) Plasma steroid-binding proteins. *Endocrinology and Metabolism Clinics of North America* **20**: 697-720.

Ross, J.B.A., Contino, P.B., Lulka, M.F. and Petra, P.H. (1985) Observation and quantitation of metal-binding sites in the sex steroid-binding protein of human and rabbit sera using the luminescent probe terbium. *J Pro Chem* **4**: 299-304.

Sairam, M.R. and Bhargavi, G.N. (1985) A role for glycosylation of the α subunit in transduction of biological signal in glycoprotein hormones. *Science* **229**: 65-67.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**: 5463-5467.

Savu, L., Benassayag, C., Vallette, G., Christeff, N. and Nunez, E. (1981) Mouse α_1-fetoprotein and albumin: A comparison of their binding properties with estrogen and fatty acid ligands. *J Biol Chem* **256**: 9414-9418.

Scatchard, G. (1949) The attractions of proteins for small molecules and ions. *Ann N Y Acad Sci* **51**: 660-672.

Schmid, M., Feichtinger, W., Deubelbeiss, C. and Weller, E. (1987) The fragile site (17)(p12): induction by AT-specific DNA-ligands and population cytogenetics. *Hum Genet* **77**: 118-121.

Schmidt, W.N., Taylor, C.A.,Jr. and Danzo, B.J. (1981) The use of a photoaffinity ligand to compare androgen-binding protein (ABP) present in rat sertoli cell culture media with ABP present in epididymal cytosol. *Endocrinology* **108**: 786-794.

Schwalbe, R., Dahlbäck, B., Hillarp, A. and Nelsestuen, G. (1990) Assembly of protein S and C4b-binding protein on membranes. *J Biol Chem* **265**: 16074-16081.

Schwarz, H.P., Fischer, M., Hopmeier, P., Batard, M.A. and Griffin, J.H. (1984) Plasma protein S deficiency in familial thrombotic disease. *Blood* **64**: 1297-1300.

Shafie, S.M. and Liotta, L.A. (1980) Formation of metastasis by human breast carcinoma cells (MCF-7) in nude mice. *Cancer Lett* **11**: 81-87.

Siiteri, P.K., Murai, J.T., Hammond, G.L., Nisker, J.A., Raymoure, W.J. and Kuhn, R.W. (1982) The serum transport of steroid hormones. *Recent Prog Horm Res* **38**: 457-510.

Siiteri, P.K. (1986) Plasma steroid-binding proteins - past, present and future. In: *Binding Proteins of Steroid Hormones*, John Libbey & Company Ltd., London. pp. 593-610.

Skinner, M.K. and Fritz, I.B. (1985) Testicular peritubular cells secrete a protein under androgen control that modulates Sertoli cell functions. *Proc Natl Acad Sci USA* **82**: 114-118.

Skinner, M.K. and Griswold, M.D. (1983) Sertoli cells synthesize and secrete a ceruloplasmin-like protein. *Biol Reprod* **28**: 1225-1229.

Smith, D.B. and Johnson, K.S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. *Gene* **67**: 31-40.

Soule, H.D. and McGrath, C.M. (1980) Estrogen responsive proliferation of clonal human breast carcinoma cells in athymic mice. *Cancer Lett* **10**: 177-189.

Strel'chyonok, O.A., Avvakumov, G.V. and Akhrem, A.A. (1984a) Pregnancy-associated molecular variants of human serum transcortin and thyroxine-binding globulin. *Carbohydr Res* **134**: 133-140.

Strel'chyonok, O.A., Avvakumov, G.V. and Survilo, L.I. (1984b) A recognition system for sex-hormone-binding protein-estradiol complex in human decidual endometrium plasma membranes. *Biochim Biophys Acta* **802**: 459-466.

Strel'chyonok, O.A. and Avvakumov, G.V. (1990) Specific steroid-binding glycoproteins of human blood plasma: Novel data on their structure and function. *J Steroid Biochem* **35**: 519-534.

Sui, L-M., Cheung, A.W.C., Namkung, P.C. and Petra, P.H. (1992) Localization of the steroid-binding site of the human sex steroid-binding protein of plasma (SBP or SHBG) by site-directed mutagenesis. *FEBS Lett* **310**: 115-118.

Sullivan, P.M., Petrusz, P., Szpirer, C. and Joseph, D.R. (1991) Alternative processing of androgen-binding protein RNA transcripts in fetal liver. *J Biol Chem* **266**: 143-154.

Sullivan, P.M., Wang, Y-M. and Joseph, D.R. (1993) Identification of an alternate promoter in the rat androgen-binding protein/sex hormone-binding globulin gene that regulates synthesis of a messanger RNA encoding a protein with altered function. *Mol Endocrinol* **7**: 702-715.

Suzuki, Y. and Sinohara, H. (1979) Hepatic uptake of desialylated testosterone-oestradiol-binding globulin in the rat. *Acta Endocrinol* **90**: 669-679.

Szpirer, C. and Szpirer, J. (1975) A mouse hepatoma cell line which secretes several serum proteins including albumin and α-foetoprotein. *Differentiation* **4**: 85-91.

Tardivel-Lacombe, J., Egloff, M., Mazabraud, A. and Degrelle, H. (1984) Immunohistochemical detection of the sex steroid-binding plasma protein in human mammary carcinoma cells. *Biochem Biophys Res Commun* **118**: 488-494.

Thomas, P.S. (1980) Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc Natl Acad Sci USA* **77**: 5201-5205.

Tindall, D.J. and Means, A.R. (1980) Properties and hormonal regulation of androgen binding proteins. In: *Advances in Sex Hormone Research*, Volume 4. (Edited by J.A. Thomas and R.L. Singhal) Urban and Schwarzenberg, Baltimore. pp. 295-327.

Trigo-Gonzalez, G., Awang, G., Racher, K., Neden, K. and Borgford, T. (1993) Helix variants of troponin C with tailored calcium affinities. *Biochemistry* **32**: 9826-9831.

Tsai, M.-J. and O'Malley, B.W. (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* **63**: 451-486.

Van Baelen, H., Convents, R., Cailleau, J. and Heyns, W. (1992) Genetic variation of human sex hormone-binding globulin: Evidence for a worldwide bi-allelic gene. *J Clin Endocrinol Metab* **75**: 135-139.

Walsh, K.A., Titani, K., Takio, K., Kumar, S., Hayes, R. and Petra, P.H. (1986) Amino acid sequence of the sex steroid binding protein of human blood plasma. *Biochemistry* **25**: 7584-7590.

Wang, Y-M., Sullivan, P.M., Petrusz, P., Yarbrough, W. and Joseph, D.R. (1989) The androgen-binding protein gene is expressed in CD1 mouse testis. *Mol Cell Endocrinol* **63**: 85-92.

Wang, Y-M., Bayliss, D.A., Millhorn, D.E., Petrusz, P. and Joseph, D.R. (1990) The androgen-binding protein gene is expressed in male and female rat brain. *Endocrinology* **127**: 3124-3130.

Westphal, U. *Steroid-Protein Interactions II. Monographs on Endocrinology*, Heidelberg:Springer-Verlag, Berlin, 1986.

White, R., Fawell, S.E. and Parker, M.G. (1991) Analysis of oestrogen receptor dimerisation using chimeric proteins. *J Steroid Biochem Molec Biol* **40**: 333-341.

Wieslander, L. (1979) A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. *Anal Biochem* **98**: 305-309.

Wilson, J.D. (1978) Sexual differentiation. *Annu Rev Physiol* **40**: 279-306.

Wong, C.-I., Zhou, Z.-X., Sar, M. and Wilson, E.M. (1993) Steroid requirement for androgen receptor dimerization and DNA binding. *J Biol Chem* **268**: 19004-19012.

Yalcinkaya, T.M., Siiteri, P.K., Vigne, J.-L., et al. (1993) A mechanism for virilization of female spotted hyenas in utero. *Science* **260**: 1929-1931.