Molecular and Cellular Endocrinology 316 (2010) 13–23

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Review

Structural analyses of sex hormone-binding globulin reveal novel ligands and function

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ARTICLE INFO

Article history:
Received 8 June 2009
Received in revised form 4 September 2009
Accepted 4 September 2009

Keywords:
Sex hormone-binding globulin
Androgens
Estrogens
Laminin G domain

ABSTRACT

Plasma sex hormone-binding globulin (SHBG) regulates the access of androgens and estrogens to their target tissues and cell types. An SHBG homologue, known as the androgen-binding protein, is expressed in Sertoli cells of many mammalians, but testicular expression of human *SHBG* is restricted to germ cells. The primary structure of SHBG comprises tandem laminin G-like (LG) domains. The amino-terminal LG-domain includes the steroid-binding site and dimerization interface, and its tertiary structure, resolved in complex with natural and synthetic sex steroids, has revealed unanticipated mechanisms of steroid binding at the atomic level. This LG-domain interacts with fibulin-1D and fibulin-2 in a ligand-specific manner, and this is attributed to the unique way estrogens reside within the steroid-binding site, and the ordering of an otherwise flexible loop structure covering the entrance of the steroid-binding pocket. This mechanism enables estradiol to enhance the sequestration of plasma SHBG by the stroma of some tissues through binding to these extra-cellular matrix-associated proteins. The human SHBG amino-terminal LG-domain also contains several cation-binding sites, and occupancy of a zinc-binding site influences its affinity for estradiol. The complete quaternary structure of SHBG remains unresolved but structural predictions suggest that the carboxy-terminal LG-domains extend laterally from the dimerized amino-terminal LG-domains. The carboxy-terminal LG-domain contains two N-glycosylation sites, but their biological significance remains obscure. Knowledge of the SHBG tertiary structure has helped develop computational techniques based on the use of a "bench-mark data set" of steroid ligands, and created novel drug discovery and toxicology risk assessment platforms.

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doi:10.1016/j.mce.2009.09.005

1. Introduction

Sex hormone-binding globulin (SHBG) is a homodimeric plasma glycoprotein produced by hepatocytes which functions as the principal transporter of biologically active estrogens and/or androgens in the blood of all vertebrate species with the exception of birds (Hammond, 2002, 1990; Westphal, 1986; Siiteri et al., 1982; Breuner and Orchinik, 2002). The same transcription unit that encodes plasma SHBG is also expressed in the testes of most mammals, and in these species an SHBG homologue, widely known as the testicular androgen-binding protein (ABP), is produced and secreted by Sertoli cells primarily under the influence of follicle-stimulating hormone (Joseph, 1994). While the single human *SHBG* gene is also expressed in the testis (Hammond et al., 1989), human *SHBG* transcripts are confined to testicular germ cells rather than Sertoli cells, and encode an amino-terminally truncated SHBG isoform that accumulates within the acrosome of sperm and binds steroids in essentially the same way as plasma SHBG (Selva et al., 2002, 2005).

Due to its high affinity and selectivity for sex steroids, the concentration of SHBG in human blood is a major determinant of the amounts of free androgens and estrogens that enter tissues and gain access to their target cells by passive diffusion (Siiteri et al., 1982). It is also possible that plasma SHBG within the extra-cellular compartments of certain tissues can influence the biological activities of sex steroids at the target cell level (see reviews by Willnow and Nykjaer, Rosner et al. and Fortunatti et al., in this issue). What may be less well appreciated is that SHBG also binds many pharmaceutically important synthetic steroids, flavonoids and xenobiotics, and that these interactions have important implications in medicine (*e.g.*, oral contraceptives, hormone-replacement therapy, diagnostic imagining of breast cancer and diet-disease risk correlations) and environmental toxicology (*e.g.*, effects of water pollution on fish and other species). Resolving the tertiary and quaternary structure of SHBG and the precise topography of its steroid-binding site has therefore not only provided mechanistic insight into the function of this important steroid transport protein, but also has enabled the development of a computational drug discovery and environmental toxicology risk analysis platform. This review will highlight new information gained through molecular biological and X-ray crystallographic analysis within the context of a substantial body of knowledge about the biochemical properties of human SHBG, and its homologs in other species, which has been documented in a comprehensive monograph on the subject of steroid-binding proteins published in the late 1980s (Westphal, 1986), as well as in a number of other more specific review articles (Hammond, 1990; Joseph, 1994), wherein a more comprehensive list of references to numerous earlier studies can be found.

2. Biochemical properties of SHBG and its steroid-binding site

Soon after the discovery of SHBG (also known in early reports as TeBG for "testosterone/estradiol-binding globulin" and SBP for "sex steroid-binding protein") in human blood plasma, it was realized that it must circulate in the blood as a homodimeric glycoprotein (Westphal, 1986). In support of this, the hydrodynamic properties of plasma SHBG were found to be consistent with a globular protein of about 90–100 kDa when assessed by gel filtration chromatography, while the apparent molecular size of the reduced and denatured protein, as assessed by SDS-polyacrylamide gel electrophoresis, revealed the presence of two ~50 kDa molecular weight species known as the heavy (~52 kDa) and light (~48 kDa) subunits, which are present in serum SHBG in an approximately 10:1 ratio. It was not immediately accepted that these two subunits were identical polypeptide sequences, but this electrophoretic micro-heterogeneity was eventually attributed to differences in the glycosylation of a single polypeptide chain (Bocchinfuso et al., 1991). With the advent of highly specific antibodies against human SHBG, a common electrophoretic variant of SHBG with an even higher apparent molecular mass was also later identified in individual blood samples (Gershagen et al., 1987), and was found to represent a genetic variant that encodes a protein with an additional consensus site for N-glycosylation (Power et al., 1992). Although the carbohydrate chains associated with SHBG decorate a substantial portion of the carboxy-terminal regions of each SHBG subunit, they do not influence its steroid-binding activity (Bocchinfuso et al., 1991).

Early studies of SHBG in blood samples, or after its purification, indicated that its stability (in particular, stability of its dimer) was influenced by the presence of steroid ligands and metal ions, and Ca^{2+} in particular. This is an important and frequently overlooked property of SHBG, which is most clearly illustrated by the fact that SHBG is highly unstable in plasma prepared in the presence of EDTA, especially after samples are frozen and thawed. Moreover, it also became evident that removal of the steroid ligand from purified preparations of SHBG destabilized the protein, and led to the conclusion that steroids and divalent cations, such as Ca^{2+}, must play a role in dimer stability, and that dimerization was somehow required for the integrity of a single steroid-binding site per dimer (Bocchinfuso and Hammond, 1994).

Studies of the interactions between SHBG and a wide selection of natural and synthetic steroids (Westphal, 1986) also led to the conclusion that the molecular structure that best fits the SHBG steroid-binding site is the biologically active androgen, 5α-dihydrotestosterone (DHT). The planarity of the steroid molecule and particularly the angle between ring A and the B, C, D ring planes has a marked impact on the affinity of different steroids for SHBG, and a 17β-hydroxy group was found to be present in all ligands with the highest affinity for SHBG. A keto (oxygen) group at C3 appeared important for optimum binding, but a hydroxy group at this position was also well tolerated. Although the general conclusion was that optimal steroid binding to SHBG requires a planar C19 steroid with a 17β-hydroxyl and an electronegative functional group at C3, it was recognized that human SHBG also bound C18 steroids (estranes) with relatively high affinity and that additional functional groups within their phenolic ring A could substantially enhance steroid binding, and this was most apparent in the case of the catechol estrogen, 2-methoxyestradiol (Westphal, 1986).

The cloning of a cDNA and the gene encoding human SHBG (Hammond et al., 1989, 1987; Gershagen et al., 1989) confirmed the polypeptide sequence of human SHBG that was obtained at about the same time by direct amino acid sequence analysis (Walsh et al., 1986). It also provided conclusive evidence that SHBG is a homodimer of two identical 40.5 kDa polypetide sequences (subunits) that are the products of a single gene located on the short arm of human chromosome 17 (Berube et al., 1990). When compared to the cDNA-deduced sequences of SHBG homologs in the rat and several other mammalian species, as well as other proteins with related sequences, such as protein S, it became apparent that SHBG comprised two laminin G-like (LG) domains (Joseph and Baker, 1992). Although these early studies of the primary structures of SHBG in different mammalian species revealed that they were surprisingly poorly conserved, it was possible to identify highly conserved amino acids that might represent residues identified by photoaffinity-labelling experiments as interacting with specific regions of photo-labile steroid ligands, such as Δ^6-testosterone which covalently attaches to the methionine at position 139 (Met139) in the mature human SHBG sequence upon exposure to ultra-violet light (Grenot et al., 1992). Other biochemical analyses, including the use of spin-labelled steroids, indicated that the SHBG

binding pocket is rather deep, as compared to the shallower one of corticosteroid-binding globulin (Westphal, 1986). However, the location of the binding site, especially in relation to the structural organization of the homodimer, remained controversial until the analysis of recombinant molecular variants of human SHBG and the rat homologue revealed that several key residues that participated in steroid binding were clustered within their amino-terminal LG-domains (Joseph, 1994; Joseph and Baker, 1992; Hammond and Bocchinfuso, 1995). This was confirmed when it was found that this LG-domain (residues 1–194) could be expressed alone in bacteria, and that the recombinant product not only had the capacity to bind steroid in a manner that resembled the natural protein but also spontaneously homodimerized in the presence of steroid ligands (Hildebrand et al., 1995). This was the breakthrough needed for the production of sufficient amounts of a homogeneous and un-glycosylated protein that could be successfully crystallized for detailed structural analyses (Grishkovskaya et al., 1999).

3. Crystal structure of human SHBG

Although it was evident that human SHBG and its homologs in other species comprised a tandem repeat of LG-domains (Fig. 1), structural information about LG-domains remained out of reach until the crystal structures of the LG-domain from neurexin-I (Rudenko et al., 1999), two LG-domains from laminin α2 chains (LG4 and LG5) (Hohenester et al., 1999; Tisi et al., 2000) and the N-terminal LG-domain of SHBG (Grishkovskaya et al., 2000) were solved in short succession. The comparison of these crystal structures showed that LG-domains have a spherical shape and consist of two antiparallel β-sheets packed on top of each other. Of the five LG-domains present within the C-terminus of laminin α-chains, LG4 and LG5 share the highest sequence homology to the N- and C-terminal LG-domains of SHBG, respectively. A similar LG4–LG5 repeat is also present in Gas6 and protein S, two proteins involved in extra-cellular signaling and for which either a crystal structure or a homology model is available (Sasaki et al., 2006; Villoutreix et al., 2001).

In LG-domains, eight of the central β-sandwich strands, namely β-strands 1–4 and 11–14, form a so-called jellyroll motif; while strands 5–7 and 8–10 follow an "up-and-down" topology (Fig. 1A). In SHBG, only two single-turn α-helical segments are present. They are adjacent to each other and connect β-strands within the jellyroll motif. A highly conserved disulfide bridge is formed between C164 (strand 12) and C188 (strand 14) at the very C-terminus of the domain. The steroid ligand binds close to the centre of the N-terminal LG-domain of SHBG and intercalates into the hydrophobic core formed between the two β-sheets (Fig. 1A). The SHBG fold bears no resemblance to other steroid carriers, such as corticosteroid-binding globulin (see a review elsewhere in this issue), or any other plasma protein of known 3D structure that binds lipophilic molecules. Moreover, while LG-domains, including those of SHBG, are structurally related to the carbohydrate-binding pentraxins and other lectins, the location of the ligand-binding sites in LG-domains is completely different from the carbohydrate-binding sites in lectins (Rudenko et al., 2001).

Steroids are deeply buried in the interior of the N-terminal LG-domain of SHBG and form a limited number of important, sequence-specific polar interactions with the protein, along with multiple hydrophobic contacts. In case of DHT, the oxygen atom attached at C3 points into the interior of the protein and forms a hydrogen bond with the side-chain hydroxyl of Ser42 (Fig. 1C). Two additional H-bonds are formed at the entrance of the binding pocket, namely between the hydroxyl group at atom C17 and the side-chains of Asp65 and Asn82. Besides these hydrophilic anchor points, all other residues lining the steroid-binding site

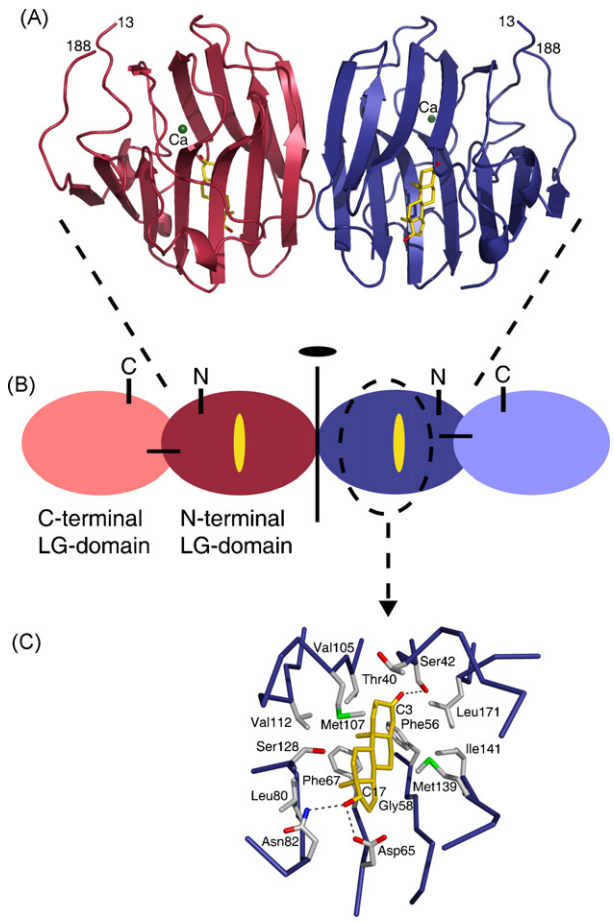

Fig. 1. Crystal structure of SHBG. (A) Ribbon representation showing how the N-terminal LG-domains (residues 13–188) of each SHBG subunit interact in a head to head manner to create a homodimer (PDB code: 1d2s) (Berman et al., 2000). The two SHBG N-terminal LG-domains, each harboring a steroid-binding site, are shown in red and blue. (B) Sketch of the domain composition of dimeric full-length SHBG. The steroids bound to the N-terminal LG-domains are depicted as yellow ellipses. The twofold symmetry axis that relates the two monomers in dimeric SHBG is indicated as a vertical line at the centre of the sketch and marked with a black ellipse. (C) Detail of the steroid-binding site: while DHT forms hydrogen bonds with SHBG via its oxygen atoms attached to C3 and C17, all additional interactions between the steroid and SHBG are restricted to hydrophobic interactions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

are hydrophobic. The main contributors to the hydrophobic ligand interactions are Phe67, Met107 and Met139 (contact surface greater than 20 Å^2). Only 2% of the ligand surface is accessible to the solvent: rings A and B are completely buried and only atoms C12 and C17 from rings C and D, respectively, are partially solvent-accessible in the original DHT complex crystal structure (Grishkovskaya et al., 2000). Next to the ligand pocket, two additional binding sites are present in SHBG, namely a calcium-binding site (site I shown in Fig. 1A) and a second metal-binding site (site II shown in Fig. 2B) whose nature only became apparent after soaking SHBG-crystals with $ZnCl_2$ (Avvakumov et al., 2001). Site II is located at the entrance to the steroid-binding site, and Zn^{2+} is coordinated by the side-chains of His83 and His136 and the carboxylate group of Asp65 (Fig. 2), and its occupancy by zinc was found to alter the specificity of steroid binding by SHBG. The affinity of SHBG for estradiol is reduced in the presence of 0.1–1 mM Zn^{2+}, whereas its affinity for DHT remains unaffected. This altered binding specificity was not observed when Zn^{2+} coordination at site II was modified by substituting a glutamine for His136 (Avvakumov et al., 2001).

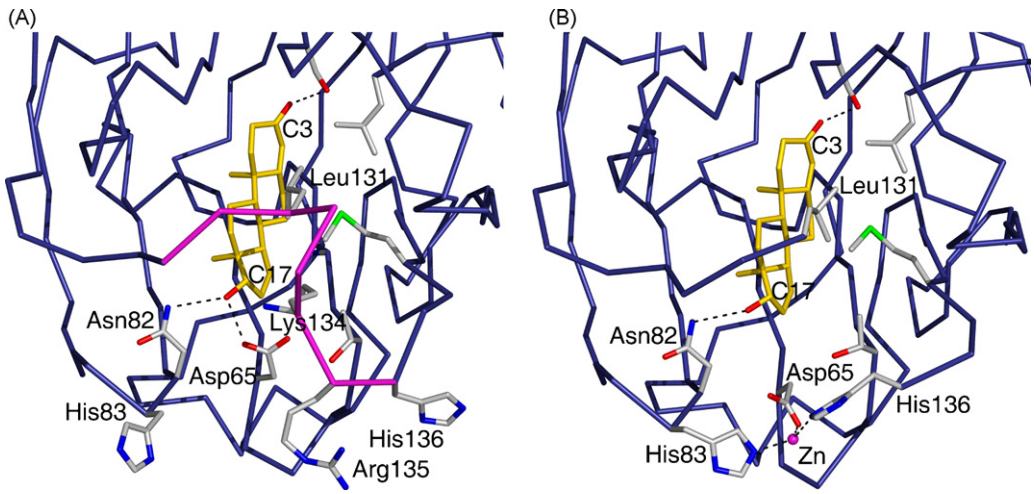

Fig. 2. Structural basis for the zinc effect in SHBG. (A) Close-up view of the SHBG steroid-binding site together with DHT bound in an orientation as in Fig. 1A (blue molecule) and Fig. 1C. In the absence of zinc the loop segments 130–135 (in magenta) covers the entrance of the steroid-binding site (PDB code:1kdk). (B) Upon zinc binding, the side-chains of residues Asp65 and His136 become reoriented. As a consequence, the loop 130–135 becomes disordered and access to the steroid-binding site is facilitated (PDB code: 1f5f). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The mechanism by which zinc ions modulate the SHBG affinity for estrogens was understood upon further structural investigations, which revealed that androgens and estrogens bind to SHBG in different orientations (see below) and that soaking DHT-bound crystals with EDTA allowed us to resolve the structure of a previously disordered region, namely residues 130–135 of SHBG (Grishkovskaya et al., 2002b). This region forms a 3_{10} helical turn that serves as a lid covering the entrance of the steroid-binding site when zinc is absent (Fig. 2A). A comparison of the structures obtained before and after soaking crystals with EDTA, as well as alterations in the apparent affinity for estradiol of SHBG variants obtained by site-directed mutagenesis (in particular, His136Gln, Asp65Ala, Gly58Ala, Pro137Gly, Pro130Gly and Asn82Ala), provided a structural model that explained the influence of zinc on SHBG-steroid interactions. In short, when zinc is absent, the Asp65 side-chain forms a hydrogen bond with the oxygen atom at C3 of estradiol, while the binding of zinc disrupts this by diverting the side-chain of Asp65 away from the steroid-binding site. Thus, this interaction with Asp65 is crucial for the high-affinity binding of estradiol to SHBG, unlike the less important interaction between Asp65 and C17 hydroxyl in the case of androgens (Fig. 2B). In addition, zinc binding reorients the side-chain of His136. In the absence of zinc, His136 points away from the SHBG surface, but when it is involved in the coordination of zinc, His136 turns inwards. This reorientation of His136 has a marked effect on the structure of the segment 130–135, as this segment becomes disordered, and this apparently interferes with the entrance of estradiol into the binding site. However, this has little or no effect on DHT, which enters the site in an opposite orientation. These effects of Zn^{2+} on the steroid-binding specificity of human SHBG may not occur in the blood circulation because most of the zinc is bound by albumin, but they may be biologically relevant within male reproductive tissues where “free” zinc concentrations are very high.

The SHBG binding modes have been structurally elucidated for a number of steroids (Grishkovskaya et al., 2002a) thereby laying the foundation for *in silico* ligand-docking studies (see below). Among the complexes studied, and as mentioned above, the structure of the N-terminal LG-domain of SHBG with estradiol yielded the most unexpected result since it showed that estradiol binds in an opposite orientation when compared to DHT (Fig. 3). Strikingly, the overall topology of the ligand interaction is however retained, with the oxygen atoms attached to atoms C3 and C17 of the respective steroids occupying almost identical positions in these complexes (Fig. 3B). The overall volume occupied by DHT and estradiol is also very similar. As a consequence, the hydrophobic residues lining the pocket contribute similar extensive contacts to the binding of both steroids. The crystal structure of the N-terminal LG-domain of SHBG in complex with levonorgestrel showed that this synthetic progestin is accommodated in the steroid-binding site in the same orientation as DHT (Fig. 3C). In the complex, the ethinyl group protrudes towards the entrance of the binding site, and this explains how SHBG can serve as a transport molecule for synthetic progestins substituted at atom C17 of the steroid ring D. In contrast to levonorgestrel, the contraceptive estrogen, ethinylestradiol, binds very poorly to human SHBG (Westphal, 1986; Pugeat et al., 1981), and this is most likely because this synthetic steroid binds in the reverse orientation compared to levonorgestrel, and its ethinyl group at C17 clashes with residues deep within the steroid-binding pocket. However, the biologically active estrogen metabolite 2-methoxyestradiol also clearly binds preferentially in the same orientation as estradiol (Avvakumov et al., 2002), with its methoxy group at C-2 forming an additional interaction with the side-chain of Asn82 at the entrance of the binding site (Fig. 3D). The latter ligand complex also helps understand the high affinity of 2-halogenated steroids for SHBG, and this may be relevant in the design of synthetic steroids whose ability to bind to SHBG might influence their pharmacological actions (Seimbille et al., 2002).

Irrespective of the type of steroid or its orientation within the SHBG steroid-binding pocket, the anchoring of ligand within the binding site of SHBG relies on hydrogen-bonding at both ends (C3 and C17) of the steroids with the same key amino acids, and in this context the ligand-binding site of SHBG resembles those of the nuclear hormone receptors, including the androgen receptor (Askew et al., 2007). However, ligand binding to SHBG does not exert such a marked conformational change in the ligand-binding pocket, when compared with the re-orientation of helix 12 of the ligand-binding domain of the nuclear hormone receptors, like the androgen receptor, which appears to firmly trap the ligand in its binding site (Matias et al., 2000). This difference probably explains why the association rates of SHBG and the sex steroid receptors for their cognate ligands are similar, while the dissociation of steroids from SHBG occurs much more rapidly than from the receptors, and this is consistent with their very different modes of action.

Unlike other proteins containing a tandem LG-domain structure, SHBG forms a homodimer in solution. Moreover, the prevailing

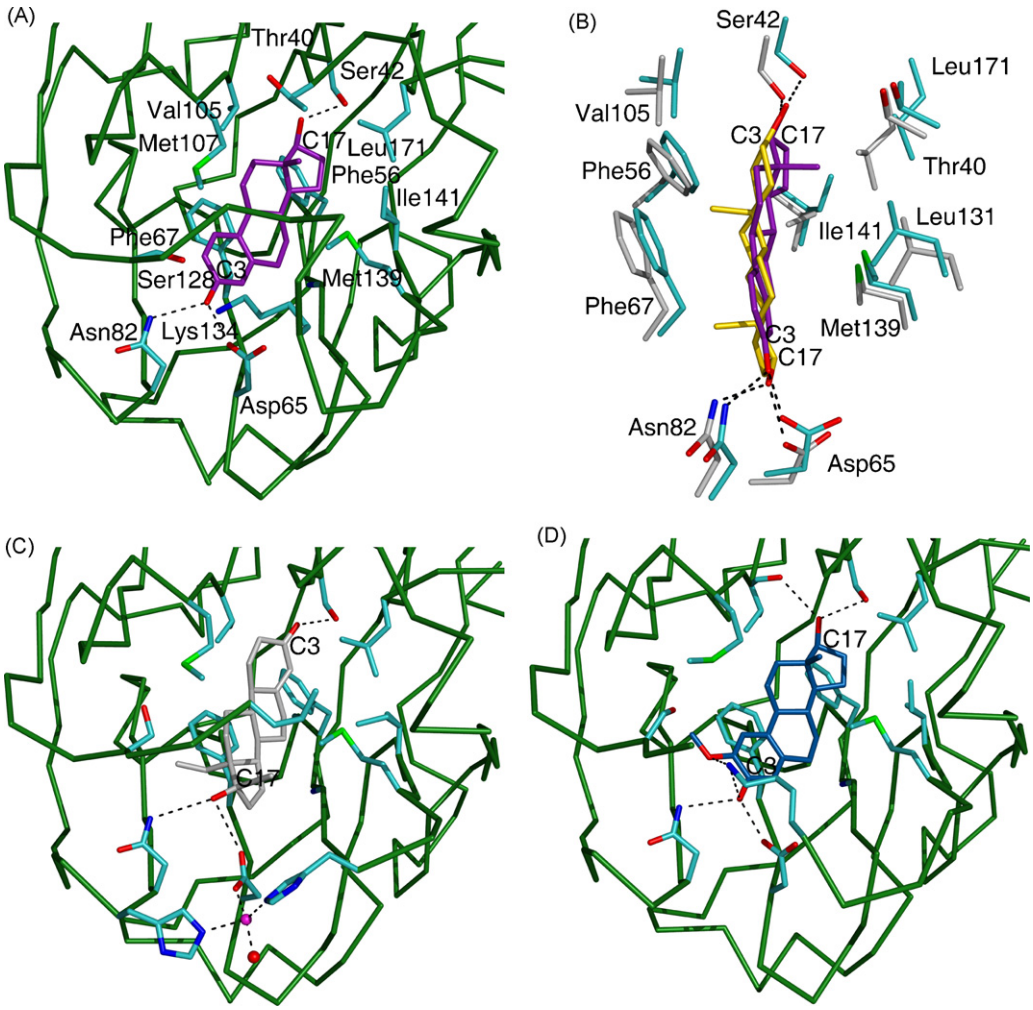

Fig. 3. Crystal structures of the N-terminal LG-domain of SHBG in complex with various steroids. (A) SHBG in complex with estradiol (PDB code: 1lhu, estradiol shown in magenta). (B) The comparison of the estradiol and DHT complex structures reveals that the two steroids bind in opposite orientations to the binding site. Estradiol is shown in magenta and the surrounding residues in cyan in the estradiol complex, whereas in case of the DHT complex, DHT is shown in yellow and the surrounding residues in light grey. (C) Crystal structure of SHBG in complex with norgestrel showing the position of a zinc atom in site II (PDB code:1lhv, norgestrel in grey, zinc atom in magenta) and (D) crystal structure of SHBG in complex with 2-methoxyestradiol (PDB code: 1lhw, 2-methoxyestradiol in dark blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

assumption in case of SHBG was that steroid-binding pocket and dimerization interface partially overlap, and that a homodimeric SHBG molecule contains only a single steroid-binding site at the dimer interface. Our studies (Avvakumov et al., 2001) have shown that the dimerization and steroid-binding sites are distinct and that both monomers within a homodimeric complex are capable of binding steroids (Fig. 1). In the SHBG dimer, the N-terminal LG-domains of each monomer interact in a head to head manner, and the interface between the N-terminal LG-domains of each monomer is very hydrophobic and involves phylogenetically conserved residues (Avvakumov et al., 2001). Moreover, because the C-termini of the two N-terminal LG-domains extend sidewise upon their dimerization (residues 188 in Fig. 1A), a simple model for full-length SHBG can be envisioned (Grishkovskaya et al., 2000), in which the C-terminal LG-domains would be placed to the left and right of the dimerized N-terminal LG-domains (Fig. 1B).

The dimerization mode was validated using site-directed mutagenesis to create full-length SHBG variants in which single amino acid substitutions (Val89Glu and Leu122Glu) were introduced in order to produce steric clashes at critical positions within the proposed dimerization domain, and this approach resulted in production of dimerization-deficient SHBG variants (Avvakumov et al., 2001). Interestingly, these dimerization-deficient SHBG variants bound steroids with an affinity and specificity indistinguishable from the wild-type SHBG. Moreover, when equalized in terms of their monomeric subunit content, the dimerization-deficient and wild-type SHBGs had essentially identical steroid-binding capacities. Thus, it was unequivocally demonstrated that the human SHBG homodimer contains two equally active steroid-binding sites which raised some interesting issues related to the biological function of SHBG. It is possible, for instance, that the two steroid-binding sites within the SHBG homodimer are partially occupied or even occupied by different types of steroid ligands. These possibilities need to be considered particularly in the context of ligand-dependent interactions between SHBG and plasma membrane proteins, which have been proposed to play a role in the non-genomic mechanism of steroid hormone action (see reviews elsewhere in this issue).

Alignment of SHBG sequences from multiple animal species (Supplementary Fig. 1) shows that, although the overall sequence homology is low, most of the residues participating in the formation of the steroid-binding pocket are located within conserved polypeptide stretches. The residues that interact with oxygen atoms at the opposite ends of the steroid molecule (Ser42 and Asp65) as well as Gly58 and Phe67 are invariably present in the

same positions in SHBG sequences of all mammalian and fish species. However, other residues that are important for steroid binding to human SHBG differ, and this in part accounts for differences in the binding specificity of SHBG in different species. For instance, one of the two methionines that form extensive hydrophobic contacts with the ligand molecule in human SHBG (Met139) is replaced by a residue with a different hydrophobic side-chain in many mammals and all fish. The other methionine, Met107, is even less conserved and is substituted with a hydrophilic serine in fish (asparagine in flounder). Another important human SHBG residue (Asn82), which is involved in interactions with the aromatic-ring hydroxyl of estrogens, is highly conserved in mammals (Grishkovskaya et al., 2002a). In fish SHBGs, this latter residue is replaced by a lysine, and it appears to contribute to the much higher affinity of fish SHBG for androstenedione when compared to SHBG in mammals (Thorsteinson et al., 2009). Although the Val105 in human SHBG is also highly conserved in other mammalian SHBG sequences, it does not contact directly with steroids, and its substitution with a leucine in zebrafish SHBGs likely allows the ethinyl group of ethinylestradiol to be accommodated within the binding site (Thorsteinson et al., 2009). In addition, variability of the residues within the loop at the entrance to the steroid-binding site (Pro130–Arg135 in human SHBG) also likely influences the fine-tuning of the binding site topology to accommodate specific sex hormones in a particular species.

4. Structure of the carbohydrate moiety and biological implications of SHBG glycosylation

Unlike corticosteroid-binding globulin (see Lin et al. in this issue), the carbohydrate content of SHBG is rather low (10–12% by weight). In human SHBG, an *O*-linked glycan is attached to Thr7 and two *N*-linked chains of N-acetyllactosamine type are present in the C-terminal LG-domain at Asn351 and Asn367 (Walsh et al., 1986; Avvakumov et al., 1983; Hammond et al., 1986). Consensus sites for N-glycosylation are present in the same or similar locations in most other mammalian species except horse, but extra consensus sites present in some of them (Supplementary Fig. 1). By contrast, the glycosylation patterns of SHBG from fish species are completely different and tend to be clustered in the N-terminal LG-domain (Supplementary Fig. 1).

The structures of the carbohydrate chains of human SHBG are typical of mammalian serum glycoproteins (Hammond and Bocchinfuso, 1995) and their primary functions are likely related to the maintenance of the overall glycoprotein structure and protection of the polypeptide from proteases as well as metabolism. The *O*-linked chain is a Gal-GalNAc disaccharide that can be sialylated at one or both monosaccharide units. The *N*-linked chains can be of bi-antennary or tri-antennary variety. This, together with the partial utilization of the consensus sites and variable amounts of terminating sialic acid residues present in both *O*-linked and *N*-linked sugar chains, contributes to the micro-heterogeneity of this glycoprotein (Hammond and Bocchinfuso, 1995). Although the precise biological functions of the SHBG carbohydrates are not known, the integrity of the SHBG carbohydrate chains is important for the interaction of SHBG–steroid complexes with the plasma membranes of steroid-target cells (Strel'chyonok and Avvakumov, 1990), and *O*-glycosylation was found to be essential for the SHBG binding to a membrane receptor that is responsible for inhibiting the estradiol-induced proliferation of MCF-7 breast cancer cells (Raineri et al., 2002).

As mentioned above, the carbohydrate chains associated with SHBG are not involved in steroid binding, but they prolong the plasma half-life of the protein (Cousin et al., 1999) and may influence the biological activities of SHBG in different physiological compartments through modification of its biological clearance and possible interactions with other macromolecules. For instance, the electrophoretic micro-heterogeniety of SHBG in plasma *vs* testis homogenates is quite distinct with a reversal in relative abundance of the "heavy" and "light" glycoforms in the testis homogenates (Cheng et al., 1985). Whether this represents a difference in the glycosylation of protein that is produced in the testis *vs* the liver, or reflects a preferential sequestration of one plasma SHBG glycoform over another by specific compartments within the testis, remains to be determined.

5. Interaction of SHBG with extra-cellular matrix-associated proteins

Studies in mice that express human *SHBG* transgenes have revealed information about their temporal and spatial expression in cell types other than hepatocytes (Jänne et al., 1998). However, these *in vivo* studies clearly indicated that some tissue compartments must sequester SHBG from the blood circulation because they lack SHBG mRNA and therefore the capacity to synthesize SHBG locally: two very clear examples of this are the stromal compartments of the endometrium and epididymis (Ng et al., 2006). The obvious question then was how is SHBG sequestered from the blood plasma into these tissue compartments, and does this process involve passive or facilitated transfer mechanisms? To address this and the broader question of how SHBG might interact with other macromolecules, including membrane receptors (Rosner et al., 1999), molecular biological approaches have been used to identify proteins that interact with human SHBG, and generally these have involved yeast-2-hybrid screens (Ng et al., 2006; Pope and Lee, 2005). Because numerous reports had indicated that SHBG interacts with plasma membranes of prostate cells (see Rosner et al. in this issue), these screens involved the use of a human prostate cDNA library of expressed protein tags as the "prey library" of target amino acid sequences. Although none of these screens identified candidates for the plasma membrane-associated receptors that have been reported to interact with SHBG on human prostate and breast cancer cells, two independent screens identified the C-terminal domains of two members of the fibulin family of extra-cellular matrix-associated proteins, fibulin-1D and fibulin-2 as SHBG-interacting amino acid sequences (Ng et al., 2006). The C-terminal regions of fibulin-1D and fibulin-2 share limited sequence identity but both bind the N-terminal LG-domain of SHBG very selectively in a steroid ligand-dependent manner. For example, they do not recognize the C-terminal LG-domain of SHBG, but these fibulin family members are able to selectively bind to SHBG in the context of the complex mixture of proteins present in a serum sample, and their ability to do this is enhanced when the SHBG steroid-binding site is occupied by a ligand, and by estradiol in particular (Ng et al., 2006). More importantly, human SHBG was not only shown to co-localize with the members of the fibulin family within the stromal matrix of the endometrium of mice that express human *SHBG* transgenes, but its sequestration from the plasma within this location could be enhanced in a steroid ligand-dependent manner, such that occupancy of the human SHBG steroid-binding site with estradiol resulted in preferential endometrial uptake in pharmacologically manipulated animals (Ng et al., 2006). Moreover, in untreated transgenic mice the amounts of human SHBG in the endometrial stroma fluctuates throughout the estrus cycle in relation to the plasma concentrations of estradiol (Ng et al., 2006).

The steroid ligand dependence of the interaction between SHBG and fibulin-1D and fibulin-2 is of interest from a structural point of view because it suggests that the highly ordered configuration of the loop structure, which covers the entrance of the steroid-binding

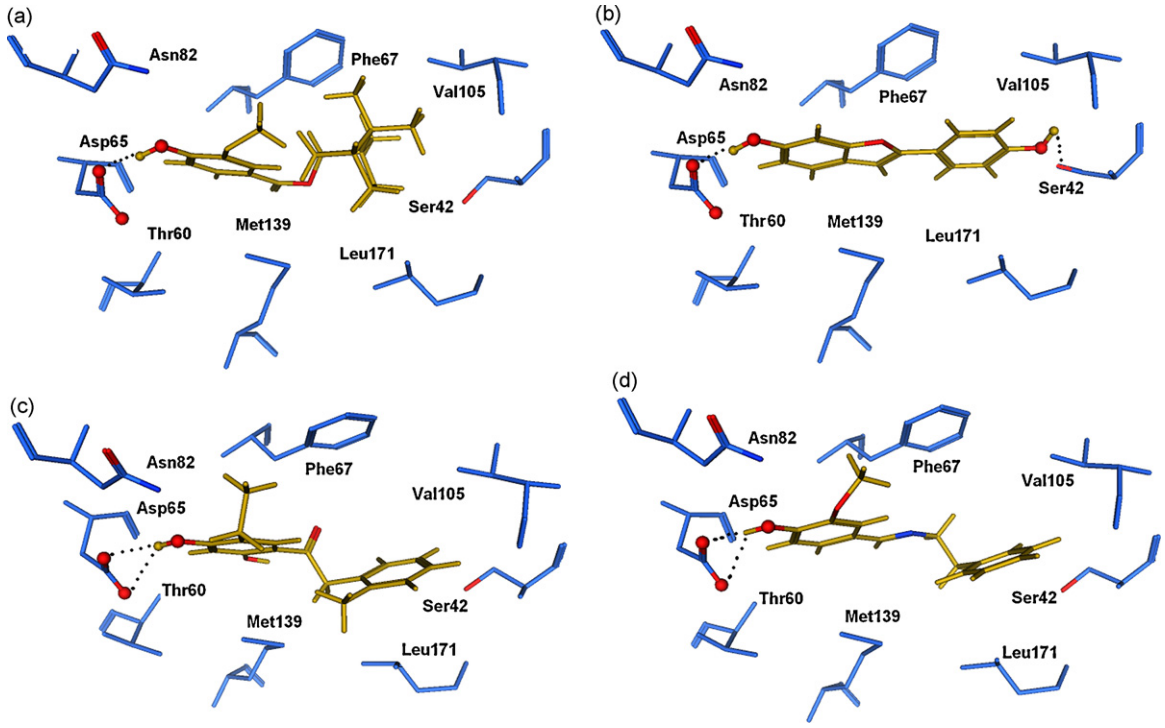

Fig. 4. Docked poses of the most active non-steroidal ligands within the SHBG steroid-binding pocket. Only those residues that are most relevant to ligand binding are shown. Hydrogen bonds are represented as white dots; hydrophobic interactions featured by thick green lines. The following four compounds (see ZINC database (Irwin and Shoichet, 2005) for compound nomenclature) are shown (ordered a–d from left to right and top to bottom in the figure): ZINC00073647 (pKa = 6.39), ZINC00334865 (pKa = 6.08), ZINC00389056 (pKa = 6.97), ZINC00084751 (pKa = 6.23). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

pocket when it is occupied by estrogens, could possibly create a stable interaction interface that is recognized preferentially by these fibulin family members. Moreover, occupancy of the SHBG binding site by androgens, which is associated with disorder of this same loop structure (Grishkovskaya et al., 2002a), appears to limit the interaction of SHBG with fibulin and the sequestration of SHBG by endometrial stroma (Ng et al., 2006), and it is possible that synthetic progestins that bind human SHBG with high affinity, and the same orientation as androgens, could limit the sequestration of SHBG by the endometrial stroma of women taking these synthetic progestins in oral contraceptive formulations.

6. *In silico* modeling and discovery of SHBG ligands

In addition to being a subject of extensive biochemical analyses and a potentially attractive drug discovery target, human SHBG also serves as a model system for conventional *in silico* chemical research. In particular, the association constants of human SHBG with a series of steroids that form part of a 'steroid benchmark set' has been used in a variety of molecular modeling studies (Polanski and Walczak, 2000), especially in the context of developing popular *in silico* modeling tools including comparative molecular field analysis (CoMFA) (Cramer et al., 1989) and molecular similarity indices in a comparative analysis (CoMSiA) (Klebe et al., 1994). In this context, the availability of nine crystal structures of the N-terminal LG-domain of human SHBG in complex with different steroid ligands not only revealed how small structural variations in these ligands accounted for their binding affinity constants, but also forced a re-evaluation of the use of the 'steroid benchmark set' for modeling studies to take into account unexpected steroid-class specific differences in their orientation within the steroid-binding site.

In our own *in silico* modeling studies of SHBG-ligand binding, we first developed several pharmacophore models for SHBG ligands and used them to screen an electronic collection of natural compounds for novel non-steroidal compounds (Cherkasov et al., 2005a). A pharmacophore represents a set of space-positioned molecular features determining the ability of a ligand to bind to its biological target, and pharmacophore models can thus be developed by superimposing the molecular structures of specific ligands against their known affinities for the same target. This allows the common structural features responsible for binding interactions to be defined, and we have developed several pharmacophore models (Cherkasov et al., 2005a) based on the flexible alignment of a large number of known ligands of human SHBG (Westphal, 1986). When these pharmacophores were applied to a collection of 23,836 'drug-like' natural substances they identified a number of non-steroidal compounds. The twenty-two top-ranked substances were tested empirically as SHBG ligands in a competitive ligand-binding assay (Hammond and Lahteenmaki, 1983), and eight of them (corresponding to four different molecular scaffolds) demonstrated activity with up to low micro-molar equilibrium dissociation constants (Cherkasov et al., 2005a).

We then applied this new information about novel non-steroidal SHBG ligands to a follow-up study that focused on the use of docking methods in drug discovery (Cherkasov et al., 2005b). In the latter work, we applied our own method for calculating partial atomic charges in proteins, and conducted a comparative docking study involving human SHBG and the steroid benchmark set. This further confirmed the superior performance of our method over several conventional systems, and resulted in identification of additional non-steroidal ligands of SHBG.

The further combination of QSAR and docking methodologies resulted in the development of a novel and powerful method of high throughput 'in silico' discovery that we have called Pro-

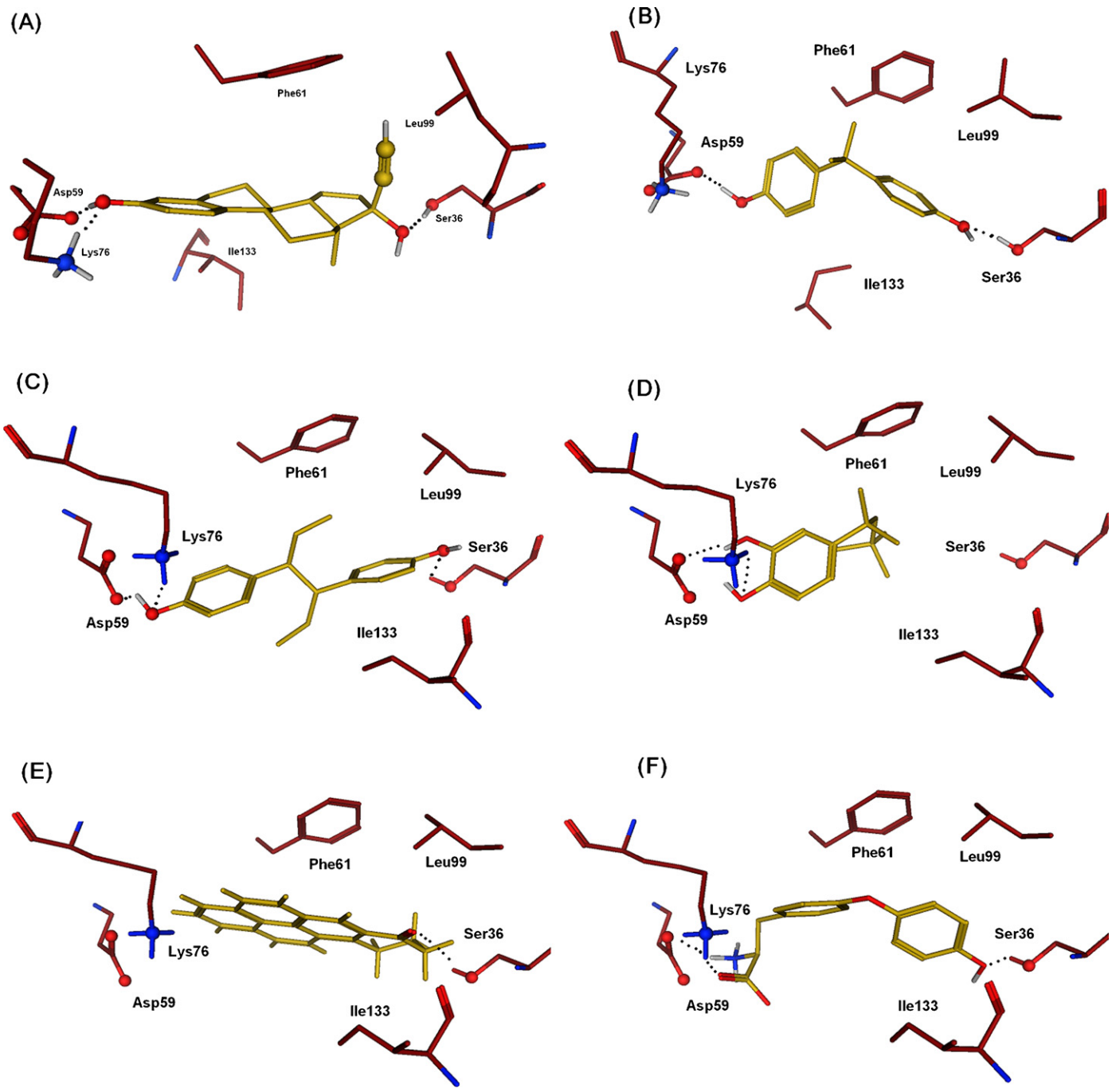

Fig. 5. *In silico* modeling of the zebrafish SHBG steroid-binding site explains its high-affinity interactions with ethinyl-estradiol and aids the identification of xenobiotic fish SHBG ligands. (A) The optimal positioning of ethinylestradiol predicted by *in silico* modeling allows hydrogen-bonding between the hydroxy-group at C3 of the steroid A ring and Lys76 as well as the highly conserved Asp59, and accommodation of ethinyl group at C17 of the steroid D ring due to the presence of Leu99 in place of Val105 in human SHBG. The positions of several xenobiotics within the zebrafish SHBG ligand-binding site, as predicted by docking experiments, are shown in panels B–F: they include: (B) bisphenol A, (C) hexestrol, (D) 4-tert-octylcatechol, (E) dihydrobenzo(a)pyren-7(8H)-one, and (F) DL-thyronine.

gressive Docking (Cherkasov et al., 2006). This approach utilizes docking scores generated for validated ligands to build predictive QSAR models that, in turn, assess hypothetical target-binding affinities for undocked entries. Most importantly, we demonstrated that 'Progressive Docking' reduces the amount of computations by 2–3-fold, and substantially accelerates high throughput screening. When applied to SHBG this new approach allowed us to identify four other novel, low micro-molar SHBG ligands.

The synergy between ligand-based (such as QSAR) and structure-based (docking) drug discovery methods has been further exploited in our work, where the updated SHBG ligand data set was used to develop a novel 4D QSAR approach (Cherkasov et al., 2008). This method relied on new ligand-induced active site descriptors (called 'inductive' 4D QSAR parameters) derived from the protein-ligand interface. The resulting structure-activity solutions not only brought additional insights into structural requirements for a ligand for effective interaction with SHBG, but also allowed quantification of the impact of various residues within its ligand-binding site. The latter approach has been applied to more than 3.3 million commercially available chemicals (Cherkasov et

al., 2008) and allowed identification of 41 potential SHBG binders. When evaluated experimentally, 25 out of 41 selected candidates demonstrated binding to human SHBG in plasma, and in some cases they bound SHBG with higher affinity than the most active non-steroidal ligands identified to date.

Analysis of the docking poses of the four most active ligands identified by *in silico* modeling has provided additional insight into the SHBG ligand-binding site (Fig. 4). As illustrated, all four ligands likely form a hydrogen bond with the Asp65 side-chain (supported by the secondary H-interaction with Asn82), and one of them (compound ZINC00334865; see ZINC database (Irwin and Shoichet, 2005) for compound nomenclature) also likely forms strong H-binding with Ser42 (supported by additional interaction with the Val105 backbone oxygen). These observations further illustrate the importance of Asp65 or Ser42 as anchoring residues, as indicated above. It should be noted, however, that the presence of two anchoring H-bonds did not make the compounds better binders. In fact, three out of four featured substances formed only one hydrogen bond, but actually demonstrate higher affinity toward the target, most likely because of more favorable hydrophobic interactions. It should be noted, however, that the affinities of these non-steroidal ligands are 2–4 orders of magnitude lower than that of estradiol, and DHT (Cherkasov et al., 2008).

Thus, our *in silico* analyses have not only established various structural determinants of SHBG–ligand interaction and facilitated the development of a number of new computational screening tools, but have also significantly expanded the set of known SHBG ligands: all of which can be used to fine-tune computational discovery tools for further *in silico* studies of SHBG in humans and other species. The practical importance of these studies extends from drug discovery to the identification of both natural and anthropogenic compounds present within the environment, and which may bind and compete with natural steroids for the SHBG steroid-binding site. For example, building on studies of human SHBG, we have applied *in silico* drug discovery methods to identify potential ligands for SHBG in zebrafish (*Danio rerio*) as a model aquatic organism (Thorsteinson et al., 2009). This is important because SHBG in fish binds xenobiotics including endocrine disruptors that have adverse effects on reproduction (Sumpter, 1998). One such compound is ethinylestradiol that binds SHBG in fish with exceptionally high affinity and is very efficiently taken up by fish from the aquatic environment through interaction with SHBG in the gills (Miguel-Queralt and Hammond, 2008). Since aquatic species are in intimate contact with this and other anthropogenic compounds, fish SHBGs represent interesting targets for bio- and chem-informatic studies of potentially harmful environmental contaminants that might pose health risks to humans and other vertebrates including fish.

Despite the fact that the sequence conservation between human and fish SHBG is less than 30%, we have been able to use our computational methods (homology modeling, molecular dynamics simulations, virtual screening, and 3D QSAR analysis) to create a model of the zebrafish SHBG steroid-binding site (Thorsteinson et al., 2009). This model explains why fish SHBG binds ethinylestradiol with high affinity, while human SHBG does not (Fig. 5A), and has allowed us to successfully identify a number of environmentally significant non-steroidal substances that bind to zebrafish SHBG with low-micro-molar to nanomolar affinities, including: bisphenol A, hexestrol, 4-tert-octylcatechol, dihydrobenzo(a)pyren-7(8H)-one, and DL-thyronine (Fig. 5B–F, respectively). This illustrates the feasibility of creating other models of fish SHBGs including models of the newly discovered salmonid SHBG-α and SHBG-β paralogs that have distinct steroid-binding specificities and sites of expression (Miguel-Queralt et al., 2009; Bobe et al., 2008).

7. Conclusions and perspectives

Resolution of the tertiary structure of the N-terminal LG-domain of SHBG has revealed exactly how steroid ligands are accommodated within a well-defined hydrophobic binding pocket, and how ligand binding alters the configuration of a highly flexible loop structure that appears to cover the entrance of the binding site. The impact that these ligand-specific differences in the conformation of this loop structure may have on the steroid-binding kinetics of androgens vs estrogens remains to be defined. It has not yet been possible to obtain a crystal structure of the N-terminal LG-domain of SHBG in the absence of steroid ligand, most likely because occupancy of the steroid-binding site stabilizes the homodimerization of the protein that occurs via pairing of two LG-domains. However, the discovery that mutations in the N-terminal LG-domain dimer interface that prevent homodimerization do not disrupt steroid binding (Avvakumov et al., 2001), suggests that it might be possible to obtain a structure of the un-liganded protein by crystallizing this type of dimer-deficient mutant. Obtaining such a structure should provide important insight into whether the protein undergoes a conformational change upon occupancy of the steroid-binding site that might influence the binding kinetics of different classes of steroids. This is also important because the information we have at present suggests that there are subtle differences in some surface residues of the protein, such as the flexible loop structure that covers the steroid-binding site, when the protein is occupied by estrogens as opposed to androgens, and this might help explain the ligand-dependency of interactions between SHBG and extracellular matrix-associated proteins, like fibulin-1D and fibulin-2. Defining the structural basis of how SHBG interacts with these and other proteins like the endocytotic receptor, megalin (Hammes et al., 2005), could help define the biological significance of such interactions through the creation of SHBG variants that lack the appropriate protein interaction sites. It is also important to appreciate that our understanding of the structure of the carboxy-terminal LG-domain of SHBG is limited to assumptions based on homology modeling, and it may interact with other macromolecules in a manner that is unrelated to steroid binding but which may be influenced by its glycosylation status. Defining these interactions might therefore be as important in terms of understanding the function of the protein, as understanding its steroid-binding site, and may reveal new ways of modifying its biological actions.

Although we know the structural requirements for SHBG homodimerization, the crystal structure of the full-length SHBG (i.e., N-terminal and C-terminal LG-domains) has not been obtained yet, and thus the complete quaternary structure of SHBG remains to be resolved. The difficulty here has been to get sufficient quantities of the full-length human SHBG in a stable dimeric conformation. It is therefore interesting that the recently discovered salmonid SHBG-β paralog does not dimerize and it may be easier to obtain a full-length structure of this protein. Obtaining the crystal structures of the two salmonid SHBG paralogs might also be very important because it could provide insight into how these two proteins interact differently with specific steroid ligands, as well as xenobiotic ligands that might be present in the aquatic environment. This information will also allow further development of *in silico* screening methods for toxicological assessments.

Acknowledgements

We thank Nathalie Pilkington for editorial support and Benedikt Schmid for help with a number of figures. This work was supported by a grant (MOP-15261) from the Canadian Institutes of Health Research (to G.L.H), and a grant (Mu1477/5) from Deutsche Forschungsgemeinshaft (to Y.A.M.). A.C. acknowledges financial

support from the Prostate Centre at the VGH/UBC, G.L.H. holds a Tier I Canada Research Chair in Reproductive Health.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mce.2009.09.005.

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