The EMBO Journal Vol.19 No.4 pp.504–512, 2000

Crystal structure of human sex hormone-binding globulin: steroid transport by a laminin G-like domain

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Human sex hormone-binding globulin (SHBG) transports sex steroids in blood and regulates their access to target tissues. In biological fluids, SHBG exists as a homodimer and each monomer comprises two laminin G-like domains (G domains). The crystal structure of the N-terminal G domain of SHBG in complex with 5α-dihydrotestosterone at 1.55 Å resolution reveals both the architecture of the steroid-binding site and the quaternary structure of the dimer. We also show that G domains have jellyroll topology and are structurally related to pentraxin. In each SHBG monomer, the steroid intercalates into a hydrophobic pocket within the β-sheet sandwich. The steroid and a 20 Å distant calcium ion are not located at the dimer interface. Instead, two separate steroid-binding pockets and calcium-binding sites exist per dimer. The structure displays intriguing disorder for loop segment Pro130–Arg135. In all other jellyroll proteins, this loop is well ordered. If modelled accordingly, it covers the steroid-binding site and could thereby regulate access of ligands to the binding pocket.

Keywords: calcium binding/dimerization/jellyroll fold/plasma protein/sex steroids

Introduction

Sex steroids play key roles in the regulation of fertility, reproduction and sexual behaviour. They exert their actions at the cell surface through a variety of poorly defined non-genomic mechanisms (Revelli *et al*., 1998) and via specific nuclear receptor proteins that function as transcriptional regulators (Mangelsdorf *et al*., 1995). Because of the pharmacological importance of steroids and related molecules, these nuclear hormone receptors have been the focus of numerous structural and functional investigations (Tanenbaum *et al*., 1998). In contrast, there is limited knowledge about the structure of the extracellular proteins that transport and modulate the access of steroid hormones to their target tissues.

Human sex hormone-binding globulin (SHBG, alternative name: sex steroid-binding protein, SBP) is a homodimeric plasma glycoprotein that binds both androgens and oestradiol with nanomolar affinities (Siiteri *et al*., 1982; Westphal, 1986). Plasma SHBG is produced primarily in hepatocytes, and a differently glycosylated isoform (Cheng *et al*., 1985), androgen-binding protein (ABP), is produced in the Sertoli cells of the testis (French and Ritzen, 1973). The gene encoding SHBG is also expressed in several other tissues, but the nature of its protein products in these tissues remains to be clarified (Joseph, 1994; Hammond and Bocchinfuso, 1995).

How sex steroids enter cells has never been defined completely. According to the free hormone hypothesis, intracellular steroid concentrations are regulated by the free diffusion of steroids across cell membranes (Mendel, 1992), and extracellular proteins serve to provide steady-state concentrations of free hormones outside cells. However, membrane binding sites for SHBG on sex steroid target tissues (Rosner, 1990) may enhance passive hormone uptake by sequestration of SHBG–steroid complex to the cell surface (Avvakumov, 1991), or promote endocytosis of the entire SHBG–steroid complex (Porto *et al*., 1995). Furthermore, the possible physiological role of SHBG has been extended by observations that it triggers cAMP-dependent signalling through binding to specific cell surface receptors in prostate (Nakhla *et al*., 1994) and MCF-7 breast cancer cells (Fortunati *et al*., 1996). The activity of the SHBG receptor appears to be regulated by occupancy of the SHBG steroid-binding site (Rosner, 1990), but its identity remains obscure.

Human SHBG is a homodimer, and each monomer comprises 373 amino acid residues (Walsh *et al*., 1986; Hammond, 1993) and consists of a tandem repeat of laminin G-like domains (Figure 1). Only the N-terminal G domain (residues 1–194) in SHBG is needed for steroid binding and the formation of a homodimer (Hildebrand *et al*., 1995). The two G domains in SHBG share little sequence homology, but together they share overall sequence similarities with other tandem G domains, and constitute a unit that is often referred to as the SHBG-like domain (Figure 1). This domain is present in the ligands of the Tyro-3 receptor protein-tyrosine kinase family, i.e. the growth arrest-specific protein 6 (GAS6) and protein S, as well as in the G domain repeat 4 and 5 of the extracellular matrix protein laminin α-chain (Joseph and Baker, 1992). Recently, GAS6 and protein S have been implicated in sperm maturation because null mutations in the Tyro-3 family receptors result in defective spermatogenesis (Lu *et al*., 1999). Protein S acts in addition as an anticoagulant cofactor of protein C and binds tightly to complement protein C4b in plasma (Villoutreix *et al*., 1997). In both GAS6 and protein S, the SHBG-like domain mediates their biological function (Mark *et al*., 1996; He *et al*., 1997). The G domains of laminin bind to the dystroglycan receptor on muscle cells and anchor the actin

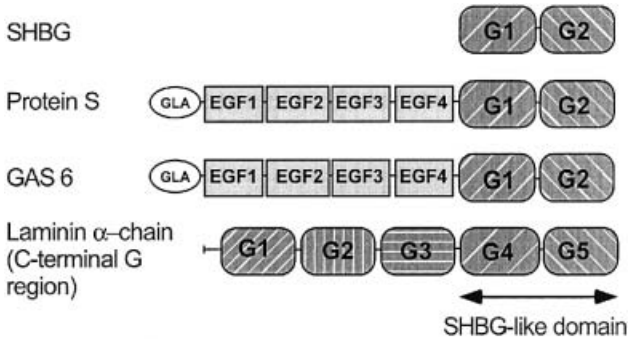

Fig. 1. Modular architecture of SHBG and its homologues GAS6, protein S and laminin. The SHBG-like domain consists of a tandem repeat of laminin G-like domains. GAS6 and protein S both contain an N-terminal GLA domain and four EGF-like domains. The domain architecture of the laminin α-chain is complex (Beck *et al*., 1990). Within each protein, the G domains share little sequence identity, and the fragment of SHBG analysed here, namely the N-terminal G domain, is homologous to the N-terminal G domain of GAS6, protein S and the fourth G domain of the laminin α-chain.

cytoskeleton to the extracellular matrix (Hemler, 1999). They also mediate the attachment of *Mycobacterium leprae* to Schwann cells and thus are implicated in the neural tropism of leprosy (Rambukkana *et al*., 1997). Additionally, G domains are present in the receptor-like neuronal cell surface molecule neurexin, the *Drosophila* developmental proteins, Slit and Crumb, and the *Drosophila* tumour suppressor Fat (Mahoney *et al*., 1991; Joseph, 1997). Thus, the functions associated with G domains are diverse and include cell signalling, adhesion, migration and differentiation.

Until recently, insights into the functional significance of G domains have been hampered by a lack of structural data. The recently solved structures of the ectodomain of neurexin-1β (Rudenko *et al*., 1999) and G domain 5 of laminin (Hohenester *et al*., 1999) revealed a close structural similarity between G domains and pentraxins (Emsley *et al*., 1994). In addition, the high resolution crystal structure of the N-terminal G domain of SHBG in complex with 5α-dihydrotestosterone (5α-DHT) presented here reveals how the same scaffold is used for a completely different function, namely for sex steroid transport in plasma.

Results and discussion

Overall structure: the laminin G-like domain fold

The crystal structure of the N-terminal G domain of human SHBG has been solved using a truncated form comprising residues 1–205 of the mature polypeptide (Walsh *et al*., 1986; Hildebrand *et al*., 1995). The structure was refined to a crystallographic *R*-factor of 20.5% (R_{free} = 25.1%) for all reflections between 40 and 1.55 Å resolution. Ninety percent of all residues are located in the most favourable regions of the Ramachandran plot and no residues are in disallowed regions (Laskowski *et al*., 1993). The domain has a spherical shape with a diameter of ~40 Å, and consists of two seven-stranded, antiparallel β-sheets packed on top of each other (Figure 2). Eight of these strands, namely β-strands 1–4 and 11–14, form a jellyroll motif (Stirk *et al*., 1992), whereas strands 5–7 and 8–10 connect to their immediate neighbours following up and down topology. Only two single

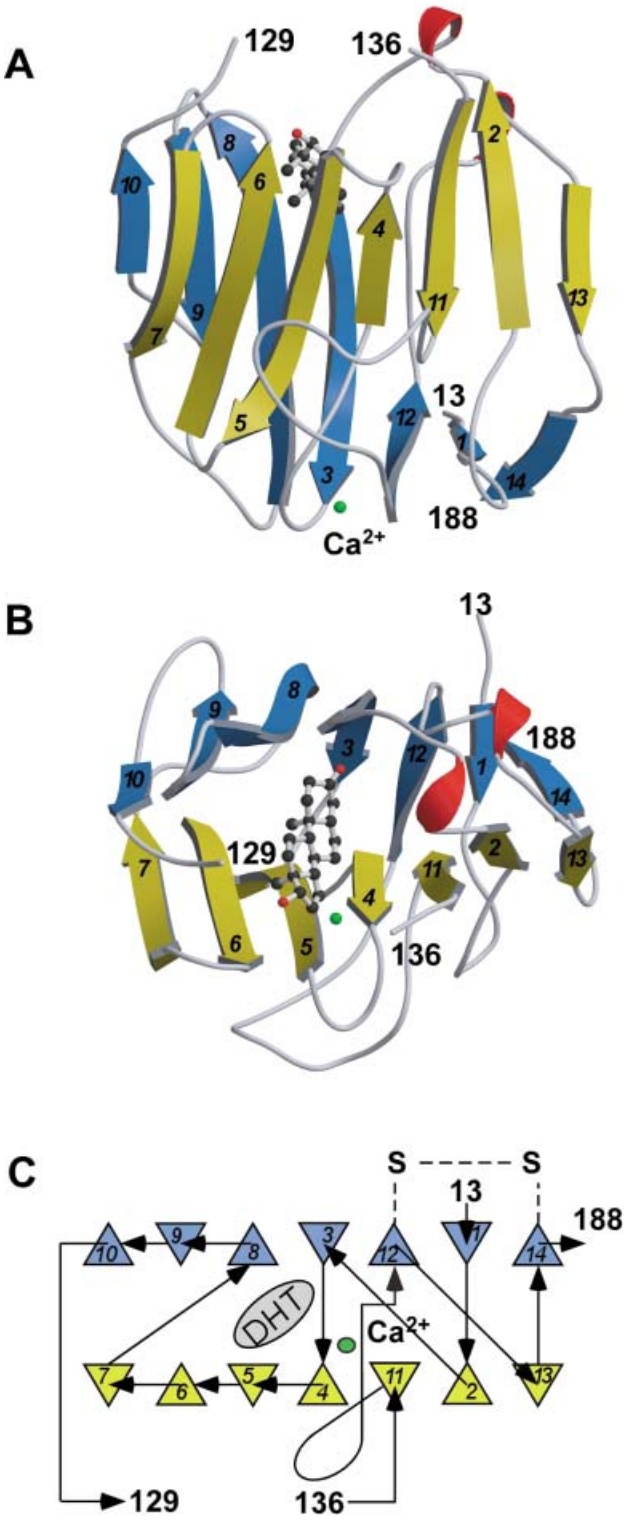

Fig. 2. The G domain fold in SHBG. (**A** and **B**) Ribbon representation of the N-terminal domain of SHBG in two orthogonal orientations. The β-strands of the two sheets forming a β-sandwich are coloured in yellow and blue, respectively. The steroid 5α-DHT is shown in a ball and stick representation. The segment 130–135, which is expected to loop over the steroid-binding pocket, is disordered and not visible in the electron density. The calcium ion is shown as a green dot (figures generated with MOLSCRIPT and RASTER3D; Kraulis, 1991; Merritt and Murphy, 1994). (**C**) Topology diagram of the β-strands. Triangles pointing upwards denote β-strands pointing out of the paper plane. The conserved disulfide bond between residues 164 and 188 is indicated as a dashed line.

turn α-helical segments are present. They are adjacent in space and connect strands within the jellyroll motif. A 3_{10} helical turn is present in the 15 residue long insertion that connects strands 11 and 12. Only one connection, namely the inter-sheet crossing between strands 10 and 11, could not be located in the electron density. A highly conserved disulfide bridge present in all G domains is formed between Cys164 from strand 12 and the C-terminal residue Cys188 from strand 14 (Figure 2C). Beyond this residue, no density was observed for the remaining 17 residues, which include the linker region connecting the N- and C-terminal G domains in the complete SHBG monomer. Likewise, no density was observed for the 12 N-terminal residues.

Searching the Protein Data Bank reveals that the structure of the N-terminal G domain of SHBG is closely related to the pentraxin serum amyloid P component (SAP) (Emsley *et al*., 1994). Among all C_α positions in both structures, 150 can be superimposed with a root mean square deviation (r.m.s.d.) of 3.0 Å. This relatively high deviation results mainly from small differences in the overall orientation of the two β-sheets in the β-sandwich. Both proteins share the jellyroll fold common to a number of functionally diverse proteins, such as neuraminidases, glucanases and lectins (Stirk *et al*., 1992). The fact that G domains are highly related to SAP was revealed recently by the determination of the crystal structure of the ectodomain of neurexin-1β (Rudenko *et al*., 1999). This corroborated previous predictions based on consensus sequence string searches, which related both the G domain and the unresolved thrombospondin N fold to pentraxin (Beckmann *et al*., 1998). However, there are several significant differences between SHBG and SAP. The N-terminal G domain of SHBG, as well as the ectodomain of neurexin-1β, lacks the long α-helix connecting β-strands 12 and 13 in SAP. Furthermore, the location of both steroid- and calcium-binding sites in SHBG differs significantly from the location of the sugar- and calcium-binding sites in SAP and lectins (Emsley *et al*., 1994). In SHBG, these binding sites are located opposite each other on the rim of the β-sheet sandwich, whereas the sugar- and calcium-binding sites in SAP and the lectins are located on identical faces of the same β-sheet. Finally, different regions on the surface mediate oligomer formation in SHBG, SAP and lectins, such as concanavalin A (Hardman and Ainsworth, 1972).

The SHBG fold bears no resemblance to albumin (He and Carter, 1992) or any plasma protein of known three-dimensional structure that binds lipophilic molecules such as steroids, thyroid hormones or retinoids. The retinol- and retinoic acid-binding proteins share an eight-stranded β-barrel and are members of the lipocalin protein family (Banaszak *et al*., 1994). Thyroxin is transported in blood by transthyretin (pre-albumin) and thyroxin-binding globulin. The monomers in tetrameric transthyretin consist of two four-stranded β-sheets (Blake and Oatley, 1977), while sequence analyses classify thyroxin-binding globulin (Flink *et al*., 1986) and corticosteroid-binding globulin (Hammond *et al*., 1987) in the serine proteinase inhibitor (serpin) superfamily. Thus, with the exception of proteins of the serpin superfamily, which have a significant proportion of α-helical structures (Huber and Carrell, 1989), these structurally dissimilar plasma hormone-binding proteins have a predominantly β secondary structure, as opposed to the almost entirely α-helical ligand-binding domains that characterize the intracellular hormone receptors (Tanenbaum *et al*., 1998).

When compared with other structural modules of extracellular proteins, such as FNIII, immunoglobulin and epidermal growth factor (EGF) domains, G domains appear to be unique because of the close proximity of the N- and C-termini. Whereas the other domains can be arranged easily like pearls on a string, a repeat of several G domains connected by short linkers will always cluster around a common point, and form an arrangement like the leaves of a clover plant. This presumably causes steric problems for the spatial accommodation of domains adjacent to the G domains and might hinder these domains in protein–protein interactions. Thus, the use of G domains as general building blocks in extracellular proteins might be limited.

Dimerization of SHBG

In blood, SHBG exists as a homodimer (Westphal, 1986) and, although the crystallographic analysis allows for the observation of a single G domain, analysis of crystal packing reveals a plausible model for homodimer formation. In this model, the monomers are related by a 2-fold crystallographic symmetry axis parallel to the β-strands, and this places β-strand 7 of one monomer next to the β-strand 10 of the second monomer, and vice versa (Figure 3A). A total of eight main chain hydrogen bonds are formed within the interface. As a consequence, the two β-sheets present in the monomers extend across the interface to form two continuous 14-stranded β-sheets. The interface area is as large as 760 $Å^2$, as expected for a permanent dimer with respect to the size of the single G domain (Jones and Thornton, 1996). All other packing contacts only range from 50 to 340 $Å^2$. The contact area is very hydrophobic in nature and involves phylogenetically invariant residues Ala85, Leu87, Val89, Leu122 and Leu124, as well as conserved residues Ala91 and Val121 (Hammond, 1993). This hydrophobic patch is surrounded by a number of highly polar interactions including two symmetry-related salt bridges between Asp73 and Arg125.

Three additional observations support the proposed dimer as a biologically relevant entity. First, ultracentrifugation experiments showed that SHBG is dimeric under the crystallization conditions (Grishkovskaya *et al*., 1999). Secondly, the identical dimer was observed in a tetragonal crystal form of SHBG, while all other packing contacts differ between the two crystal forms (data not shown). Thirdly, the distribution of atomic displacement factors supports the proposed dimerization model. The expected picture, with the lowest displacement factors (thermal mobility) in the core of the protein, and higher displacement factors at the surface of the protein, is only obtained when considering the proposed dimer as the appropriate conformation (Figure 3A).

It has been estimated that only a single steroid molecule is bound per SHBG homodimer (Westphal, 1986), and it has been proposed that this could be achieved by the formation of a single steroid-binding site by two monomeric units (Petra *et al*., 1986; Sui *et al*., 1996). Other data have suggested that the steroid-binding sites of each monomer are located in close proximity to the dimerization

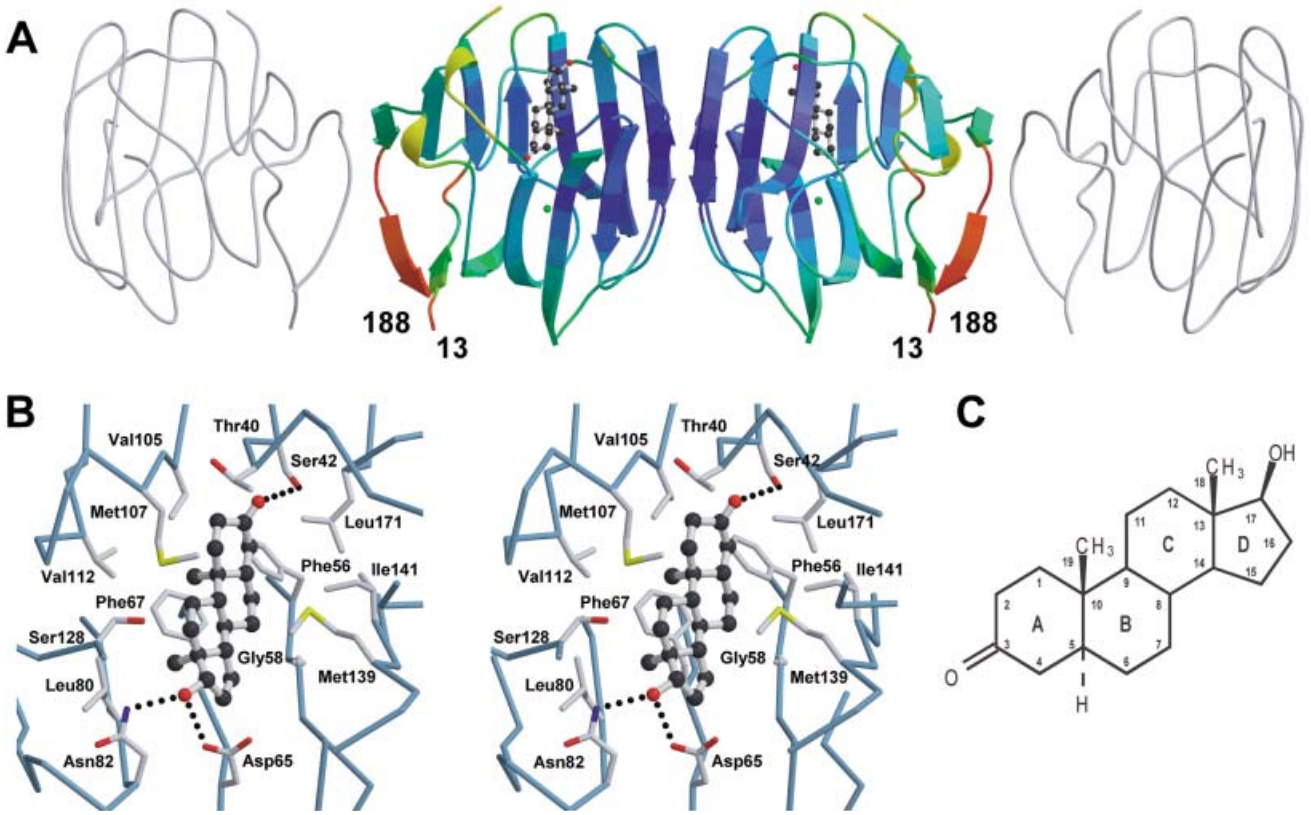

Fig. 3. (**A**) Ribbon representation of the human SHBG dimer coloured according to the atomic displacement factors (temperature factors) and model for the packing of the C-terminal G domains (in grey). The displacement factors range from 12 (dark blue) to 55 Å^2 (red). (**B**) Stereo representation of the steroid-binding pocket in an orientation identical to that in Figure 2B. All side chains that are in contact with the steroid are displayed. (**C**) Chemical structure and atom numbering in 5α-DHT. In testosterone, a double bond is present between atoms C4 and C5. In oestradiol, ring A is aromatic, C19 is missing and the carbonyl oxygen at C3 is replaced by a hydroxyl group.

interface (Bocchinfuso and Hammond, 1994), and in this case occupancy of one site might sterically hinder entry of steroid into the second site. However, in the observed crystal structure, the steroid-binding sites are 24 Å apart; they do not participate in the dimerization interface and are fully occupied. The reason for this is not immediately obvious, but the very high concentrations of steroid used during crystal growth may have saturated both sites artificially. If so, this would obscure any physiologically relevant long-range allosteric rearrangements that limit access of ligand to one of the binding sites upon occupancy of the other. As an example of this type of mechanism, transthyretin undergoes an allosteric rearrangement upon thyroxin binding, whereby the transthyretin tetramer binds a first molecule of thyroxin with a dissociation constant of ~10 nM, and a second molecule then binds with a thousand times lower affinity (Nilsson *et al*., 1975).

Dimer formation may occur in the absence of calcium (Sui *et al*., 1996), but divalent cations, such as Ca^{2+} and Zn^{2+}, stabilize the dimer (Bocchinfuso and Hammond, 1994). Human SHBG contains two calcium-binding sites per monomer (Ross *et al*., 1985), but only one is present in the N-terminal G domain. Calcium is coordinated to residues from two loop segments, namely to the carboxylate group of the side chain of Asp50 and the carbonyl oxygens of Glu52 from the loop connecting β-strands 3 and 4, and Ala160 from the loop between β-strands 11 and 12, as well as to three solvent molecules. In the sequence, Asp50 is close to residues participating in the steroid-binding site, but the calcium ion is 20.5 Å from the closest steroid atom and thus does not participate directly in steroid binding. The calcium-binding site is also distant from the dimer interface. Therefore, Ca^{2+} can only promote dimer formation (Bocchinfuso and Hammond, 1994; Hildebrand *et al*., 1995) indirectly through the overall stabilization of the G domain resulting from the cross-linking of presumably otherwise more flexible loop regions.

A model for full-length SHBG

The N-terminal G domain of human SHBG is responsible for its primary biological function, namely steroid transport in the blood (Hammond, 1997), but the C-terminal G domain might make minor contributions to steroid binding and dimer formation. Sequence alignments show that the linker between the N- and C-terminal G domains is only about five residues long. This greatly restricts possible orientations of this second G domain and prevents it from making contacts with regions of the N-terminal G domain involved in steroid binding. This is supported by previous observations that the steroid-binding affinities of C-terminally truncated forms of SHBG, which contain only the N-terminal G domain, are similar to or even higher than that of full-length SHBG (Hildebrand *et al*., 1995). Although orientations are possible in which the C-terminal G domain could participate in the dimer interface, we favour a model that places the C-terminal G domains of each subunit to the left and right of the

dimerized N-terminal G domains (Figure 3A), and which predicts that full-length SHBG has a stick-like appearance with dimensions of ~40 Å × 160 Å. This structure corresponds well to the elongated cylindrical shape of full-length SHBG derived from its hydrodynamic behaviour in size exclusion chromatography experiments, as well as the short rods of SHBG molecules with dimensions of 30 ± 10 Å × 190 ± 30 Å in electron micrographs (Beck *et al*., 1997).

The steroid-binding site

The steroid ligand 5α-DHT intercalates into the hydrophobic core of the SHBG N-terminal G domain in between two β-sheets (Figure 3B). Its oxygen atom at C3 of ring A points into the interior of the protein and is anchored to Ser42, which is strictly conserved among species (Hammond, 1993). Only 2% of the 5α-DHT surface is accessible to solvent in the complex. The partially accessible atoms are C12 and C17 from rings C and D of the steroid, respectively, while rings A and B are completely buried. The steroid-binding pocket is lined by residues contributed from eight β-strands, and is predominantly hydrophobic. The main binding contributions are from Phe67, Met107 and Met139 (contact surface >20 Å^2). Two additional hydrophilic contacts are formed between the hydroxyl group at C17 and the strictly conserved residues Asp65 and Asn82. The conservation of Gly58 also appears to be important because any side chain at this position would generate steric clashes with atoms C6 and C7 of the steroid. The conformation of 5α-DHT does not change when bound to SHBG. Its 19 carbon and two oxygen atoms can be superimposed on the crystal structure of unbound 5α-DHT with an r.m.s.d. of 0.11 Å.

The structure of the human SHBG steroid-binding pocket is in agreement with mutagenesis data (Bocchinfuso *et al*., 1992; Sui *et al*., 1992; Bocchinfuso and Hammond, 1994) and photolabelling experiments (Grenot *et al*., 1992; Kassab *et al*., 1998). In particular, it explains why the substitution of Ser42 with leucine causes a complete loss of steroid binding with no apparent effect on the dimerization potential of intact human SHBG expressed in CHO cells (Hammond and Bocchinfuso, 1996). In the structure, atom C6 of steroid ring B and the sulfur atom of Met139 are only 4.5 Å apart, and photoaffinity labelling with Δ^6-testosterone or Δ^6-oestradiol specifically marks Met139 (Grenot *et al*., 1992). The relative importance of residues in contact with the steroid is also emphasized by their across-species conservation (Hammond, 1993). Strictly conserved are residues Ser42, Phe56, Gly58, Asp65, Phe67, Asn82, Met139 and Leu171, whereas residues Leu80, Val105, Met107, Val112 and Ile141 can be substituted with other hydrophobic residues. Not conserved in SHBG from other species are Thr40, which is substituted by proline in rodents, alanine in rabbit and isoleucine in sheep, and Ser128, which is replaced by phenylalanine in sheep (Hammond, 1993). In the case of Thr40, where the C_β-atom is in contact with the steroid, different side chains can fulfil identical roles. However, these differences could contribute to differences in affinities of steroid ligands and explain why human SHBG has a much higher affinity for oestradiol when compared with SHBG from most other mammalian species (Renoir *et al*., 1980).

Intriguing local disorder prevents the localization of residues 130–135 in the present crystal structure. The absence of crystal packing contacts in this region rules out a crystallization artefact. Although poorly conserved, residues within this loop region, such as Lys134 and Arg135, have been photoaffinity labelled with steroid ligands (Namkung *et al*., 1990; Chambon, 1997) and have been shown to influence the relative binding affinities of different sex steroids across species (Bocchinfuso and Hammond, 1994; Danzo and Joseph, 1994). In particular, substitution of Arg135 with leucine causes an ~2-fold increase in affinity for only steroids with a 3β-hydroxyl group irrespective of the A/B ring angle (Bocchinfuso and Hammond, 1994). It is therefore conceivable that this loop modulates steroid-binding kinetics through transient sequence-specific interactions with particular ligands, and that differences in binding constants might result from conformational changes in this loop upon ligand binding. Possibly, the loop is ordered in the absence of steroids, because in all other structurally related proteins, such as neurexin-1β (Rudenko *et al*., 1999), pentraxin (Emsley *et al*., 1994) and lectins, local disorder is never observed within this segment. If built accordingly in SHBG, the loop would form a 'lid' over the steroid-binding site, and the phylogenetically conserved residue Leu131 would protrude into the hydrophobic steroid-binding pocket. According to this model, steroid binding may be associated with structural rearrangements and the loop could serve as a 'gatekeeper' that restricts access of lipophilic molecules to the binding site.

Occupancy of the SHBG steroid-binding site has also been reported to modulate its interactions with cell surface proteins (Rosner, 1990). The biological significance of these interactions is not well understood, but they may represent a novel pathway for signal transduction by sex steroid hormones. In this context, it has been proposed that unliganded SHBG first binds to a cell surface receptor, and binding of steroid to receptor-bound SHBG will cause SHBG to dissociate and thereby to trigger an increase of intracellular cAMP (Rosner, 1990). Peptide mapping has identified residues 48–57 as being involved in the binding of SHBG to its proposed membrane receptor (Khan *et al*., 1990). However, in the crystal structure, only residues 48–52 are partially exposed on the surface of ligand-bound SHBG where they participate in the formation of the calcium-binding site (Figure 2).

The immediate vicinity of the SHBG steroid-binding pocket might also represent an alternative receptor-binding site. The steroid dependency of the interaction could then be explained assuming ligand-dependent conformational changes in the disordered loop region. In neurexin-1β, the corresponding region has been identified as the ligand-binding site, and an RGD sequence maps on the same surface in G domain 3 of the laminin α-chain (Rudenko *et al*., 1999). Thus, this region might represent a preferential protein–protein interaction surface common to laminin G-like domains.

The observed intercalation of 5α-DHT between two β-sheets of a jellyroll protein is novel and differs from the α-helical ligand-binding sites in nuclear hormone receptors (Tanenbaum *et al*., 1998; Pike *et al*., 1999). However, several structures are known where steroids bind in a mixed α and β secondary structure environment. Although some of these structures are from steroidogenic

enzymes, a common feature seems to be the almost complete burial of steroid in a predominantly hydrophobic pocket with <10% of the surface remaining solvent accessible. In these structures, the steroid is usually oriented such that its oxygen atom at C3 points towards the centre of the protein. In contrast to SHBG where only a single hydrogen bond is formed involving the hydroxyl group of Ser42, an extensive network of hydrophilic contacts anchors the steroid via the oxygen at C3 in most other structures (see, for example, Kim *et al*., 1997).

Determinants of human SHBG steroid-binding affinity and specificity

Human SHBG binds testosterone, its precursor androst-5-ene-3β,17β-diol and its biologically active 5α-reduced metabolites with approximately nanomolar affinities (Westphal, 1986). It also binds oestradiol with relatively high affinity, and in this regard differs from SHBG in many other mammalian species (Renoir *et al*., 1980). It is remarkable, however, that all steroid ligands of SHBG (Westphal, 1986) differ structurally only with respect to chemical modifications at C3 and the configuration of their rings A and B (Figure 3C). Differences in their A/B ring angles primarily reflect the degree of ring A saturation, and determine the relative orientation of their hydroxyl or carbonyl oxygen atoms as potential donors or acceptors for hydrogen bonding with Ser42. The relatively modest 20-fold range in their relative affinities for the human SHBG steroid-binding site reflects the adaptability of the binding pocket within the vicinity of the A ring. This adaptability does, however, have limits: 5β-DHT binds poorly to SHBG, and here the bowing angle between ring A and the BCD plane is as large as 65° (Duax and Norton, 1975).

In contrast to rings A and B, rings C and D of all natural steroid ligands of human SHBG are conformationally and chemically identical, and any deviation from a β-hydroxyl group at C17 in the steroid ring D reduces binding affinities by several orders of magnitude. This is best exemplified by the much lower affinities of human SHBG for androstenedione and oestrone when compared with those for testosterone and oestradiol, respectively (Westphal, 1986). Thus, of the two positions where hydrogen bonding occurs between steroid ligands and structural residues within the SHBG steroid-binding site, the coordination of a β-hydroxyl group at C17 by Asp65 and Asn82 represents the major single determinant of steroid-binding affinity and specificity.

The specificity of the human SHBG steroid-binding site has been studied extensively using a large variety of steroid derivatives substituted with different functional groups at various positions (Westphal, 1986). In addition to emphasizing the primary importance of a β-hydroxyl group at C17, modifications at several other positions have been shown to alter relative binding affinities significantly. For example, the introduction of β-hydroxyl or oxo-groups at C11 in the 5α-DHT structure greatly reduces binding affinity, and in the observed crystal structure these modifications to ring C interfere with hydrophobic contacts between the steroid molecule and the side chain of Met107. In addition, an α-hydroxyl group at C16 (e.g. in oestriol) markedly reduces binding affinity, and this may be due to steric hindrance caused by the peptide bond connecting Asp65 and Trp66 (Figure 3B). Many of the synthetic steroids that are used as contraceptive progestins, such as norgestrel, bind to human SHBG with appreciable affinities and are characterized by an α-ethynyl group at C17 in association with an obligatory 17β-hydroxyl group (Westphal, 1986). The structure shows that indeed space is available for the accommodation of an α-ethynyl group at this position (Figure 3B).

Implications for protein S, GAS6 and laminin

The closest protein homologues containing an SHBG-like domain are GAS6, protein S and the G domain 4 and 5 repeat in the laminin α-chain. In GAS6, protein S and laminin, numerous biological functions have been linked directly to the SHBG-like domain (Gee *et al*., 1993; Mark *et al*., 1996; He *et al*., 1997), but it is not known whether these involve predominantly the N- or C-terminal G domain. In the case of the SHBG-like domain within laminin, peptide mapping suggests that heparin binding might be a property of G domain 5, whereas cell adhesion properties localize onto domain 4 (Skubitz *et al*., 1991). Although the functional significance of N- versus the C-terminal G domains of protein S and GAS6 remains to be defined, several conclusions can be drawn from the structure of the N-terminal G domain of SHBG with respect to the biological function of its related proteins.

First, binding of the SHBG-like domain of protein S to complement protein C4b and of laminin to dystroglycan is calcium dependent (Gee *et al*., 1993; He *et al*., 1997). The calcium-binding residue Asp50 in SHBG is conserved in protein S and GAS6 (Figure 4). It is also part of a proteolytic fragment of SHBG that shares considerable sequence similarity with protein S and GAS6 (Figure 4), and which inhibits the binding of SHBG to a specific plasma membrane receptor (Khan *et al*., 1990). Therefore, if this calcium-binding site is conserved between these three proteins, it may represent a common feature that contributes to their receptor-binding properties. In contrast, no acidic residue is located at this site within the G domain 4 of laminin, and this suggests that the calcium dependence of its binding to dystroglycan involves some other unrelated structural component. In the recent crystal structure of G domain 5 of laminin, a calcium-binding site is observed at the opposite side of the molecule, namely proximal to the steroid-binding site of SHBG (Hohenester *et al*., 1999).

Secondly, activation of receptor tyrosine kinases results from receptor dimerization upon ligand binding. All known ligands of receptor tyrosine kinases form homo- or heterodimers or dimerize in the presence of auxiliary molecules (Lemmon and Schlessinger, 1994). In contrast, transduction activation in the cytokine receptor family involves a monomeric ligand that can induce receptor dimerization because two distinct receptor-binding sites exist on the ligand (Wells and de Vos, 1996). Due to the substitution of hydrophobic residues in β-strand 7 of SHBG with hydrophilic residues in its related proteins (Figure 4), we do not anticipate that GAS6 and protein S could homodimerize, as observed for SHBG, and indeed protein S is monomeric (Villoutreix *et al*., 1997). Therefore, if GAS6 and protein S function as monomeric proteins, this raises some interesting questions about their ability to function as receptor tyrosine kinase ligands. For instance, they may heterodimerize with related proteins or bind accessory

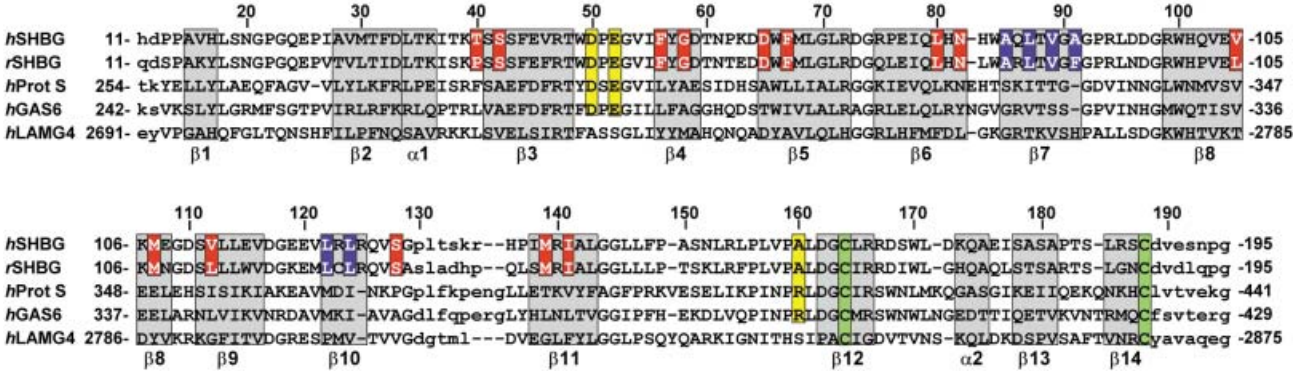

Fig. 4. Multiple sequence alignment of the first G domain in the SHBG family. Sequence identities between human and rat SHBG (*h*SHBG, *r*SHBG), human protein S (*h*Prot S), human GAS6 (*h*GAS6) and G domain 4 of the human laminin α-chain (*h*LamG4) are 71, 26, 25 and 19%, respectively. Secondary structure elements of SHBG are shown as grey boxes; residues involved in steroid binding (red), in the dimerization interface (blue), in calcium binding (yellow) and in disulfide bridge formation (green) are highlighted.

Table I. Summary of crystallographic analysis

	Native	$PtCl_4$	*cis*-$Pt(NH_3)_2Cl_2$	K_2PtCl_6	$GdCl_3$
Data collection and MIRAS analysis					
Soaking concentrations (mM)/time (h)	–	5/72	5/12	5/12	10/72
Wavelength (Å)	0.9119	1.072	1.5418	1.5418	1.5418
Resolution (Å)[a]	1.55 (1.75–1.55)	2.55 (2.6–2.55)	2.80 (3.0–2.8)	2.80 (3.0–2.8)	4.00 (4.45–4.00)
Completeness (%)[a]	96.4 (95.0)	94.3 (32.8)	84.9 (81.6)	99.2 (99.6)	91.7 (79.2)
Completeness of anomalous differences	–	93.4	71.3	–	–
R_{merge} (%)[a,b]	4.2 (23.4)	5.2 (33.6)	7.0 (17.0)	7.4 (19.1)	7.6 (7.9)
$<I/\sigma>$[a]	16.5 (3.35)	9.36 (2.16)	9.93 (4.47)	14.4 (5.14)	11.82 (9.82)
R_{iso} (%)[c]	–	21.0	20.2	17.1	19.6
No. of heavy atom sites	–	6	3	3	1
R_{Cullis} (%)[d]	–	66	62	71	81
Phasing power[e]	–	1.85	2.09	1.58	1.26
Structure refinement (40–1.55 Å)					
No. of reflections working/free	22 208/2461				
R/R_{free} (%)	20.5/25.1				
R.m.s.d. bond/angle (Å)[f]	0.013/0.030				
R.m.s.d. *B*-factors bonded atoms main chain/side chains (Å^2)	2.79/3.58				
No. of protein residues/no. of solvent molecules	170 (missing residues 1–12, 130–135, 189–205)/123				

[a]Limits and values for the outer resolution bin are given in parentheses.
[b]$R_{merge} = \Sigma\Sigma|I_{i,j} - <I_j>|/\Sigma\Sigma I_{ij}$
[c]$R_{iso} = \Sigma||F_P| - |F_{PH}||/\Sigma|F_P|$
[d]$R_{Cullis} = \Sigma||F_{PH} \pm F_P|-F_H|/\Sigma|F_{PH} \pm F_P|$, F_P, F_{PH} and F_H being the structure factor amplitudes of the native protein, the heavy atom derivative and the heavy atom contribution, respectively.
[e]Phasing power = $<|F_H|>/<IE>$, $<IE>$ being the root mean square lack of closure error.
[f]Root mean square deviation given from ideal values.

proteins in the vicinity of the receptor. It is also possible that two receptor-binding sites exist on the surface of GAS6 and protein S. If so, the mechanism by which they activate their receptors may resemble the activation of cytokine receptors rather than the mechanisms observed for receptor tyrosine kinases. Whatever scenario prevails, the jellyroll scaffold we have observed for the first time in the receptor-binding domains of these tyrosine kinase receptor ligands adds a novel biological role for this ubiquitous structural element.

Finally, it has recently been shown that spermatogenesis is defective in mice with null mutations of all three members of the Tyro-3 family of tyrosine kinase receptors that recognize GAS6 and protein S as ligands (Lu *et al.*, 1999). These observations suggest that GAS6, protein S and SHBG share a common mechanism of action that may be related to the fact that SHBG is also produced in Sertoli cells, and mediates the androgen-dependent maturation of sperm (Joseph, 1994).

In summary, the structural data presented here suggest that these proteins diverge significantly not only in their modular architecture and steroid-binding abilities but also in their dimerization behaviour. Nonetheless, it is entirely possible that variations in the tertiary and quaternary structures of cell surface receptor ligands provide for diversity in a common signalling mechanism for this family of proteins.

Materials and methods

Protein expression, purification and crystallization

Details of the expression, purification and crystallization of the N-terminal G domain of human SHBG are published elsewhere (Grishkovskaya *et al.*, 1999). In brief, a polypeptide comprising residues 1–205 of SHBG fused to GST was produced in *Escherichia coli* strain JM109 using the pGEX-2T

(Pharmacia, Uppsala) expression vector (Hildebrand *et al*., 1995). This fusion protein was isolated on a glutathione–Sepharose column, cleaved with thrombin and purified further by anion exchange and gel filtration chromatography in the presence of 3 μM 5α-DHT and 2.5 mM $CaCl_2$. Crystals were obtained using the hanging drop method, as follows: 1 μl of protein solution (13 mg/ml in 50 mM HEPES buffer pH 7.5, 2.5 mM $CaCl_2$, 3 μM 5α-DHT) was mixed together with 1 μl of reservoir solution (20% isopropanol, 10% PEG400, 100 mM HEPES buffer pH 7.5), and was equilibrated against 1 ml of the latter. The crystals belong to space group *R*32 (cell axes: a = 104.04, c = 84.43 Å, trigonal indexing). V_M = 1.9 Å^3/Da with one monomer in the asymmetric unit.

Structure determination

The crystal structure was solved with the multiple isomorphous replacement method using four different derivatives prepared by soaking the crystals in heavy atom solutions. The native data set and the $PtCl_4$ derivative were collected at the DESY synchrotron in Hamburg, the remaining data sets were collected using CuKα radiation from a rotating anode source (Table I). All data sets were collected at 100 K and reduced with XDS (Kabsch, 1988). Initial heavy atom sites were detected with HEAVY (Terwilliger *et al*., 1987) and refined with the program MLPHARE of the CCP4 (CCP4, 1994) suite of programs. Anomalous differences from two of the derivatives were included in phase calculations, resulting in an overall figure of merit of 0.56. After solvent flattening, visual inspection of the electron density at 2.6 Å resolution revealed most of the chain fold, as well as the position of the steroid. Because of the high resolution of the native data, experimental phases could be extended automatically to 1.55 Å using the free atom refinement method in ARP/WARP (Perrakis *et al*., 1997). Automatic chain tracing and subsequent sequence docking resulted in a model consisting of 122 out of 205 residues. Of those, 52% were modelled according to their correct sequence, while the remainder were modelled as alanines or serines. The model was then completed manually with O (Jones *et al*., 1991) and subjected to several cycles of refinement with REFMAC (CCP4, 1994) and manual rebuilding. The final model consists of 170 residues of which 10 side chains display alternative conformations. In addition to the steroid 5α-DHT, a calcium-binding site was identified that coincides with the binding site of Gd^{3+} in the $GdCl_3$ derivative. In final refinement rounds, a strong difference density peak remained close to the end of strand β6. Although similar in shape to a solvent molecule, this site could not be explained by water and was modelled unsatisfactorily as an additional calcium-binding site despite a very atypical coordination sphere. No individual anisotropic thermal displacement factors were refined, and the average isotropic value of the model (29.19 Å^2) agrees well with the value obtained from a Wilson plot (29.30 Å^2) (Wilson, 1949).

Acknowledgements

We thank Rolf Misselwitz and Albrecht Otto (MDC, Berlin) for help with the initial characterization of the protein, and Anna Gonzales from the EMBL outstation at DESY, Hamburg, for help with data collection. We are especially indebted to Udo Heinemann for sharing laboratory facilities, numerous discussions and, together with Wolf-Hagen Schunck, for critical reading of the manuscript. This work is supported in part by the Deutsche Forschungsgemeinschaft and the Medical Research Council of Canada. Coordinates have been deposited with the Protein Data Bank (accession code 1D2S).

References

Avvakumov,G.V. (1991) Membrane recognition of steroid–glycoprotein complexes: a model for steroid delivery to the target cells. In Gabius,H.-J. and Gabius,S. (eds), *Lectins and Cancer*. Springer-Verlag, Heidelberg, Germany, pp. 263–272.

Banaszak,L., Winter,N., Xu,Z., Bernlohr,D.A., Cowan,S. and Jones,T.A. (1994) Lipid-binding proteins: a family of fatty acid and retinoid transport proteins. *Adv. Protein Chem*., **45**, 89–151.

Beck,K., Hunter,I. and Engel,J. (1990) Structure and function of laminin: anatomy of a multidomain glycoprotein. *FASEB J.*, **4**, 148–160.

Beck,K., Gruber,T.M., Ridgway,C.C., Hughes,W., Sui,L. and Petra,P.H. (1997) Secondary structure and shape of plasma sex steroid-binding protein—comparison with domain G of laminin results in a structural model of plasma sex steroid-binding protein. *Eur. J. Biochem*., **247**, 339–347.

Beckmann,G., Hanke,J., Bork,P. and Reich,J.G. (1998) Merging extracellular domains: fold prediction for laminin G-like and amino-terminal thrombospondin-like modules based on homology to pentraxins. *J. Mol. Biol.*, **275**, 725–730.

Blake,C.C. and Oatley,S.J. (1977) Protein–DNA and protein–hormone interactions in prealbumin: a model of the thyroid hormone nuclear receptor? *Nature*, **268**, 115–120.

Bocchinfuso,W.P. and Hammond,G.L. (1994) Steroid-binding and dimerization domains of human sex hormone-binding globulin partially overlap: steroids and Ca^{2+} stabilize dimer formation. *Biochemistry*, **33**, 10622–10629.

Bocchinfuso,W.P., Warmels Rodenhiser,S. and Hammond,G.L. (1992) Structure/function analyses of human sex hormone-binding globulin by site-directed mutagenesis. *FEBS Lett*., **301**, 227–230.

CCP4 (1994) The CCP4 suite: programs for protein crystallography. *Acta Crystallogr. D*, **50**, 760–763.

Chambon,C.P. (1997) Etude de site de liaison des stéroïdes de la protéine plasmatique humaine de transport des stéroïdes ('sex hormone-binding globuline') par photomarquage d'affinité. PhD Thesis, Université Claude Bernard, Lyon, France.

Cheng,C.Y., Musto,N.A., Gunsalus,G.L., Frick,J. and Bardin,C.W. (1985) There are two forms of androgen binding protein in human testes. Comparison of their protomeric variants with serum testosterone–estradiol binding globulin. *J. Biol. Chem.*, **260**, 5631–5640.

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. (1975) *Atlas of Steroid Structure*. IFI-Plenum, New York, NY.

Emsley,J., White,H.E., O'Hara,B.P., Oliva,G., Srinivasan,N., Tickle,I.J., Blundell,T.L., Pepys,M.B. and Wood,S.P. (1994) Structure of pentameric human serum amyloid P component. *Nature*, **367**, 338–345.

Flink,I.L., Bailey,T.J., Gustafson,T.A., Markham,B.E. and Morkin,E. (1986) Complete amino acid sequence of human thyroxine-binding globulin deduced from cloned DNA: close homology to the serine antiproteases. *Proc. Natl Acad. Sci. USA*, **83**, 7708–7712.

Fortunati,N., Comba,A., Becchis,M., Catalano,M.G., Fazzari,A., Fissore,F. and Frairia,R. (1996) MCF-7 cell progesterone receptor (PGR) is additionally modulated by sex steroid binding protein (SBP) and its membrane receptor (SBP-R) through cAMP and PKA. *Ann. NY Acad. Sci*., **784**, 453–457.

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.

Gee,S.H., Blacher,R.W., Douville,P.J., Provost,P.R., Yurchenco,P.D. and Carbonetto,S. (1993) Laminin-binding protein 120 from brain is closely related to the dystrophin-associated glycoprotein, dystroglycan and binds with high affinity to the major heparin binding domain of laminin. *J. Biol. Chem.*, **268**, 14972–14980.

Grenot,C., de Montard,A., Blachere,T., de Ravel,M.R., 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 delta 6 derivatives of either testosterone or estradiol as unsubstituted photoaffinity labeling reagents. *Biochemistry*, **31**, 7609–7621.

Grishkovskaya,I., Sklenar,G., Avvakumov,G.V., Dales,D., Behlke,J., Hammond,G.L. and Muller,Y.A. (1999) Crystallisation of the N-terminal domain of human sex hormone-binding globulin, the major sex steroid carrier in blood. *Acta Crystallogr. D*, **55**, 2053–2055.

Hammond,G.L. (1993) Extracellular steroid-binding proteins. In Parker,M. (ed.), *Steroid Hormone Action*. IRL Press at Oxford University Press, Oxford, UK, pp. 1–25.

Hammond,G.L. (1997) Determinants of steroid hormone bioavailability. *Biochem. Soc. Trans*., **25**, 577–582.

Hammond,G.L. and Bocchinfuso,W.P. (1995) Sex hormone-binding globulin/androgen-binding protein: steroid-binding and dimerization domains. *J. Steroid Biochem. Mol. Biol*., **53**, 543–552.

Hammond,G.L. and Bocchinfuso,W.P. (1996) Sex hormone-binding globulin: gene organization and structure/function analyses. *Horm. Res*., **45**, 197–201.

Hammond,G.L., Smith,C.L., Goping,I.S., Underhill,D.A., Harley,M.J., Reventos,J., Musto,N.A., Gunsalus,G.L. and Bardin,C.W. (1987) Primary structure of human corticosteroid binding globulin, deduced from hepatic and pulmonary cDNAs, exhibits homology with serine protease inhibitors. *Proc. Natl Acad. Sci. USA*, **84**, 5153–5157.

Hardman,K.D. and Ainsworth,C.F. (1972) Structure of concanavalin A at 2.4 Å resolution. *Biochemistry*, **11**, 4910–4919.

He,X.M. and Carter,D.C. (1992) Atomic structure and chemistry of human serum albumin. *Nature*, **358**, 209–215.

He,X., Shen,L., Malmborg,A.C., Smith,K.J., Dahlback,B. and Linse,S. (1997) Binding site for C4b-binding protein in vitamin K-dependent protein S fully contained in carboxy-terminal laminin-G-type repeats. A study using recombinant factor IX–protein S chimeras and surface plasmon resonance. *Biochemistry*, **36**, 3745–3754.

Hemler,M.E. (1999) Dystroglycan versatility. *Cell*, **97**, 543–546.

Hildebrand,C., Bocchinfuso,W.P., Dales,D. and Hammond,G.L. (1995) Resolution of the steroid-binding and dimerization domains of human sex hormone-binding globulin by expression in *Escherichia coli*. *Biochemistry*, **34**, 3231–3238.

Hohenester,E., Tisi,D., Talts,J.F. and Timpl,R. (1999) The crystal structure of a laminin G-like module reveals the molecular basis of α-dystroglycan binding to laminins, perlecan and agrin. *Mol. Cell*, **4**, 783–792.

Huber,R. and Carrell,R.W. (1989) Implications of the three-dimensional structure of α1-antitrypsin for structure and function of serpins. *Biochemistry*, **28**, 8951–8966.

Jones,S. and Thornton,J.M. (1996) Principles of protein–protein interactions. *Proc. Natl Acad. Sci. USA*, **93**, 13–20.

Jones,T.A., Zou,J.-Y., Cowan,S.W. and Kjeldgaard,M. (1991) Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr. A*, **47**, 110–119.

Joseph,D.R. (1994) Structure, function and regulation of androgen-binding protein/sex hormone-binding globulin. *Vitam. Horm.*, **49**, 197–280.

Joseph,D.R. (1997) Sequence and functional relationships between androgen-binding protein/sex hormone-binding globulin and its homologs protein S, Gas6, laminin and agrin. *Steroids*, **62**, 578–588.

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.

Kabsch,W. (1988) Evaluation of single crystal X-ray diffraction data from a position sensitive detector. *J. Appl. Crystallogr.*, **21**, 916–924.

Kassab,D., Pichat,S., Chambon,C., Blachere,T., Rolland de Ravel,M., Mappus,E., Grenot,C. and Cuilleron,C.Y. (1998) Photoaffinity labeling of homologous Met-133 and Met-139 amino acids of rabbit and sheep sex hormone-binding globulins with the unsubstituted Delta 6-testosterone photoreagent. *Biochemistry*, **37**, 14088–14097.

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.

Kim,S.W. *et al*. (1997) High-resolution crystal structures of delta5–3-ketosteroid isomerase with and without a reaction intermediate analogue. *Biochemistry*, **36**, 14030–14036.

Kraulis,P.J. (1991) MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.*, **24**, 946–950.

Laskowski,R.A., MacArthur,M.W., Moss,D.S. and Thornton,J.M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.*, **26**, 283–291.

Lemmon,M.A. and Schlessinger,J. (1994) Regulation of signal transduction and signal diversity by receptor oligomerization. *Trends Biochem. Sci*., **19**, 459–463.

Lu,Q.X. *et al*. (1999) Tyro-3 family receptors are essential regulators of mammalian spermatogenesis. *Nature*, **398**, 723–728.

Mahoney,P.A., Weber,U., Onofrechuk,P., Biessmann,H., Bryant,P.J. and Goodman,C.S. (1991) The *fat* tumor suppressor gene in *Drosophila* encodes a novel member of the cadherin gene superfamily. *Cell*, **67**, 853–868.

Mangelsdorf,D.J. *et al.* (1995) The nuclear receptor superfamily: the second decade. *Cell*, **83**, 835–839.

Mark,M.R., Chen,J., Hammonds,R.G., Sadick,M. and Godowsk,P.J. (1996) Characterization of Gas6, a member of the superfamily of G domain-containing proteins, as a ligand for Rse and Axl. *J. Biol. Chem.*, **271**, 9785–9789.

Mendel,C.M. (1992) The free hormone hypothesis. Distinction from the free hormone transport hypothesis. *J. Androl*., **13**, 107–116.

Merritt,E.A. and Murphy,M.E.P. (1994) RASTER3D version 2.0, a program for photorealistic molecular graphics. *Acta Crystallogr. D,* **50**, 869–873.

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.

Nilsson,S.F., Rask,L. and Peterson,P.A. (1975) Studies on thyroid hormone-binding proteins. II. Binding of thyroid hormones, retinol-binding protein and fluorescent probes to prealbumin and effects of thyroxine on prealbumin subunit self association. *J. Biol. Chem.*, **250**, 8554–8563.

Perrakis,A., Sixma,T.K., Wilson,K.S. and Lamzin,V.S. (1997) WARP: improvement and extension of crystallographic phases by weighted averaging of multiple-refined dummy atomic models. *Acta Crystallogr. D*, **49**, 129–149.

Petra,P.H., Namkung,P.C., Senear,D.F., McCrae,D.A., Rousslang,K.W., Teller,D.C. and Ross,J.B. (1986) Molecular characterization of the sex steroid binding protein (SBP) of plasma. Re-examination of rabbit SBP and comparison with the human, macaque and baboon proteins. *J. Steroid Biochem*., **25**, 191–200.

Pike,A.C.W., Brzozowski,A.M., Hubbard,R.E., Bonn,T., Thorsell,A.-G., Engstrom,O., Ljunggren,J., Gustafsson,J.-A. and Carlsquist,M. (1999) Structure of the ligand-binding domain of oestrogen receptor β in the presence of a partial agonist and a full antagonist. *EMBO J.*, **18**, 4608–4618.

Porto,C.S., Lazari,M.F., Abreu,L.C., Bardin,C.W. and Gunsalus,G.L. (1995) Receptors for androgen-binding proteins: internalization and intracellular signalling. *J. Steroid Biochem. Mol. Biol*., **53**, 561–565.

Rambukkana,A., Salzer,J.L., Yurchenco,P.D. and Tuomanen,E.I. (1997) Neural targeting of *Mycobacterium leprae* mediated by the G domain of the laminin-α2 chain. *Cell*, **88**, 811–821.

Renoir,J.M., Mercier-Bodard,C. and Baulieu,E.E. (1980) Hormonal and immunological aspects of the phylogeny of sex steroid binding plasma protein. *Proc. Natl Acad. Sci. USA*, **77**, 4578–4582.

Revelli,A., Massobrio,M. and Tesarik,J. (1998) Nongenomic actions of steroid hormones in reproductive tissues. *Endocr. Rev.*, **19**, 3–17.

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

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 serum using the luminescent probe terbium. *J. Protein Chem*., **4**, 299–304.

Rudenko,G., Nguyen,T., Chelliah,Y., Südhof,T.C. and Deisenhofer,J. (1999) The structure of the ligand binding domain of neurexin-Iβ: regulation of LNS domain function by alternative splicing. *Cell*, **99**, 93–101.

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.

Skubitz,A.P., Letourneau,P.C., Wayner,E. and Furcht,L.T. (1991) Synthetic peptides from the carboxy-terminal globular domain of the A chain of laminin: their ability to promote cell adhesion and neurite outgrowth and interact with heparin and the β1 integrin subunit. *J. Cell Biol.*, **115**, 1137–1148.

Stirk,H.J., Woolfson,D.N., Hutchinson,E.G. and Thornton,J.M. (1992) Depicting topology and handedness in jellyroll structures. *FEBS Lett*., **308**, 1–3.

Sui,L.M., Cheung,A.W., 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.

Sui,L.M., Hughes,W., Hoppe,A.J. and Petra,P.H. (1996) Direct evidence for the localization of the steroid-binding site of the plasma sex steroid-binding protein (SBP or SHBG) at the interface between the subunits. *Protein Sci.*, **5**, 2514–2520.

Tanenbaum,D.M., Wang,Y., Williams,S.P. and Sigler,P.B. (1998) Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains. *Proc. Natl Acad. Sci. USA*, **95**, 5998–6003.

Terwilliger,T., Kim,S.-H. and Eisenberg,D. (1987) Generalized method of determining heavy-atom positions using the difference Patterson function. *Acta Crystallogr. A*, **43**, 1–5.

Villoutreix,B.O., Garcia de Frutos,P., Lovenklev,M., Linse,S., Fernlund,P. and Dahlback,B. (1997) SHBG region of the anticoagulant cofactor protein S: secondary structure prediction, circular dichroism spectroscopy and analysis of naturally occurring mutations. *Proteins*, **29**, 478–491.

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 plasma. *Biochemistry*, **25**, 7584–7590.

Wells,J.A. and de Vos,A.M. (1996) Hematopoietic receptor complexes. *Annu. Rev. Biochem*., **65**, 609–634.

Westphal,U. (1986) *Steroid–Protein Interactions II*. Springer-Verlag, Berlin, Germany.

Wilson,A.J.C. (1949) The probability distribution of X-ray intensities. *Acta Crystallogr.*, **2**, 318–321.

Received October 21, 1999; revised and accepted December 10, 1999