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Review

# Molecular and structural basis of steroid hormone binding and release from corticosteroid-binding globulin

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ABSTRACT

Corticosteroid-binding globulin (CBG), a non-inhibitory member of the serine proteinase inhibitor (serpin) super-family, is the high-affinity transport protein for glucocorticoids in vertebrate blood. Plasma CBG is a glycoprotein with 30% of its mass represented by N-linked oligosaccharide chains. Its well-characterized steroid-binding properties represent a "bench-mark data set" used extensively for *in silico* studies of protein–ligand interactions and drug design. Recent crystal structure analyses of intact rat CBG and cleaved human CBG have revealed the precise topography of the steroid-binding site, and shown that cortisol-bound CBG displays a typical stressed (S) serpin conformation with the reactive center loop (RCL) fully exposed from the central β-sheet A, while proteolytic cleavage of the RCL results in CBG adopting a relaxed (R) conformation with the cleaved RCL fully inserted within the protein core. These crystal structures have set the stage for mechanistic studies of CBG function which have so far shown that helix D plays a key role in coupling RCL movement and steroid-binding site integrity, and provided evidence for an allosteric mechanism that modulates steroid binding and release from CBG. These studies have also revealed how the irreversible release of steroids occurs after proteolysis and re-orientation of the RCL within the R conformation. This recent insight into the structure and function of CBG reveals how naturally occurring genetic CBG mutations affect steroid binding, and helps understand how proteolysis of CBG enhances the targeted delivery of biologically active steroids to their sites of action.


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## 1. Corticosteroid-binding globulin (CBG): a serine proteinase inhibitor (serpin) super-family member with unique biological properties

Corticosteroid-binding globulin (CBG) is a monomeric glycoprotein with a single steroid-binding site that binds as much as 90% of circulating cortisol and corticosterone in humans and rats, respectively (Hammond, 1990). Remarkably, CBG shares little sequence homology with other steroid carriers, and is defined as a clade A serine proteinase inhibitor (serpin) family member that also includes the plasma transport protein for thyroxin, thyroxin-binding globulin (TBG) (Law et al., 2006). This was first realized when it was discovered that the primary structure of CBG shares a remarkable level of sequence identity with α1-antitrypsin (AAT) and α1-antichymotrypsin (ACT), both of which are clade A serpins with well defined serine proteinase inhibitory properties (Hammond et al., 1987). By contrast, CBG and TBG do not appear to act as proteinase inhibitors but rather serve as substrates for specific proteinases like neutrophil elastase (Pemberton et al., 1988; Hammond et al., 1990a), the activity of which is normally controlled by AAT (Hammond et al., 1991).

This close relationship between CBG and AAT/ACT extends to the organization of their genes (Seralini et al., 1990), which are clustered together with 10 other related genes and pseudogenes within a serpin A gene locus of about 370 kb of genomic DNA on human chromosome 14q32.1 (Namciu et al., 2004) that exhibits a high degree of synteny between species. This serpin gene locus can be further subdivided into three sub-clusters of related genes, the most proximal of which spans about 90 kb of genomic DNA that includes the AAT (SERPINA1) gene, an AAT-related pseudogene (SERPINA2), the CBG (SERPINA6) gene, and a protein Z-dependent protease inhibitor (SERPINA10) gene (Namciu et al., 2004). The transcriptional activation of this serpin gene cluster appears to be coordinately regulated by a locus control region upstream of the AAT gene (Zhao et al., 2007) that responds to transcription factors like hepatic nuclear factor (HNF)-1 and HNF-4 involved in mediating important physiological responses, for instance in relation to inflammation (Rollini and Fournier, 1999).

The recognition that CBG and AAT are so closely related, at both the gene and protein levels, reinforces the concept that CBG likely appeared during the evolution of vertebrate species as a result of a gene duplication event (Hammond, 1990). When this occurred during evolution is unclear but it is of interest that mammals, reptiles and birds all appear to have CBG in blood, while claims that fish also have a CBG-like protein in their blood have never been substantiated (Seal and Doe, 1963; Westphal, 1986; Breuner and Orchinik, 2002). The fact that AAT and CBG are targets for the same serine proteinase, neutrophil elastase, and are both classified as "acute phase response proteins" during infectious and inflammatory diseases (Hammond et al., 1991), implies that CBG has evolved to complement the activities of AAT in the control of inflammation. This is a reasonable assumption because the steroid-binding specificity of CBG exhibits a preference for anti-inflammatory steroids, i.e., the glucocorticoids and progesterone (Hammond et al., 1991). Although CBG is produced by hepatocytes in the liver, the CBG gene is also expressed in several other tissues during development, including the kidney and pancreas (Scrocchi et al., 1993a,b). The biological significance of CBG production by these extra-hepatic tissues is not well understood, but it does not appear to contribute to plasma CBG levels and likely serves to control the tissue availability of steroids locally rather than systemically. In this context, the timing of CBG expression in organs like the kidney is of interest because it coincides with periods of active tissue remodeling involving substantial proteolytic activities (Scrocchi et al., 1993a,b).

While AAT interacts with neutrophil elastase to form a covalent one-to-one inhibitory complex after cleavage between residues designated as P1-P1′ of a surface-exposed reactive center loop (RCL) (Johnson and Travis, 1978; Carrell et al., 1982), the RCL region of CBG is simply cleaved by neutrophil elastase without forming an inhibitory complex (Pemberton et al., 1988; Hammond et al., 1990a), and the kinetics of these reactions between neutrophil elastase and AAT or CBG are extremely rapid (Brantly et al., 1988). It should also be noted that in the presence of free radicals, Met358 at P1 in the RCL of human AAT is oxidized and is resistant to neutrophil elastase cleavage at this position (Johnson and Travis, 1978; Johnson and Travis, 1979). Importantly, this prevents AAT from forming an inhibitory complex with this proteinase, and the RCL of oxidized AAT is instead cleaved by elastases at a location corresponding to the P6–P7 residues, and this essentially inactivates AAT as a proteinase inhibitor (Johnson and Travis, 1978, 1979; Carrell et al., 1982; Banda et al., 1987). A methionine is not present within the CBG RCL and its recognition by neutrophil elastase is therefore not influenced by the presence of superoxide radicals. This is considered physiologically important because the plasma concentrations of AAT are much greater that those of CBG, and oxidation of the AAT RCL at sites of inflammation would result in increased exposure of CBG to its possible attack by neutrophil elastase (Hammond, 1990).

It is well established that the cleavage of CBG by neutrophil elastase results in the irreversible destruction of the steroid-binding site, which is accompanied by a marked increase in thermo-stability and a decrease in steroid-binding affinity (Pemberton et al., 1988; Hammond et al., 1990a,b). Since most glucocorticoids in the blood are bound to CBG, this would allow a very substantial increase in the local concentrations of free glucocorticoids at specific sites of action where CBG undergoes proteinase attack. Thus, all the evidence suggests that CBG in the blood serves as a reservoir of anti-inflammatory steroids that can be rapidly released at sites of inflammation in a targeted manner (Hammond, 1990). Although this has been difficult to demonstrate *in vivo*, recent studies of a *cbg* knockout mice have indicated that these animals are much more sensitive to an acute inflammatory challenge than their wild-type counterparts (Petersen et al., 2006). This finding lends credence to earlier observations that some strains of mice, such as BC57BL/6 mice which have relatively low levels of CBG in their blood, are much more sensitive to lethal shock induced by tumor necrosis factor, and that this genetic trait is linked to the *cbg* locus (Libert et al., 1999).

Until recently, our understanding of the biochemical and molecular properties of CBG have been hampered by the lack of a tertiary structure of CBG in complex with a steroid ligand. Early attempts to obtain a CBG crystal structure through the purification of plasma CBG in complex with cortisol failed, most likely because of variations in the degree and composition of N-linked oligosaccharides that decorate the protein isolated from plasma. Expressing proteins in bacteria can circumvent this problem, but attempts to do this with human CBG have not yet met with success. The reason for this is unclear and we initially considered that this might be an impossible task because our early biochemical analyses of human CBG glycosylation-deficient mutants suggested that the utilization of a phylogenetically conserved N-glycosylation site might be important for the correct folding of CBG during synthesis, at least in mammalian cells (Avvakumov et al., 1993; Avvakumov and Hammond, 1994a). However, this may not be correct because rat CBG can be produced in bacteria in a complex with cortisol, and crystallization of this rat CBG:cortisol complex allowed us to define the structure and topography of the steroid-binding site, as well as a plausible mechanism for facilitated steroid binding and release under normal homeostatic conditions or after proteolysis of the RCL (Klieber et al., 2007). Further insight into CBG

function was obtained through structural analyses of a cleaved human CBG–AAT chimaera (Zhou et al., 2008), and a detailed study of the structural requirements for human CBG protease interactions and their functional consequences (Lin et al., 2009). The objective of this review is to assess our current knowledge of the structure of CBG in relation to its biological and functional properties.

## 2. Biochemical characterization of CBG and its steroid-binding site

### 2.1. Physicochemical properties

Plasma proteins that bind glucocorticoids and other steroid hormones have been identified in a wide range of vertebrate species, and their physicochemical properties have been studied extensively and documented in two comprehensive monographs by Westphal (1971, 1986). The most recent of these monographs was published more than two decades ago, but they still represent an invaluable resource for researchers in this field, and the following review will therefore focus on key advances made in the intervening years.

Although the amino-terminal sequence of human CBG and its amino acid composition had been defined in early studies (Westphal, 1971), the primary structure of CBG was only resolved after the isolation of a cDNA encoding the human CBG precursor polypeptide (Hammond et al., 1987). The sequence of the complete 373 residue mature human CBG polypeptide was deduced from knowledge of its amino-terminal sequence, and the most surprising feature of the CBG sequence was that it was not related to those of other steroid-binding proteins, metabolizing enzymes or receptors, but instead resembled the archetypal serpin, AAT, suggesting some unanticipated functions for CBG (Hammond et al., 1987). The availability of molecular biological tools to study the structure and biological significance of CBG, quickly led to the isolation and characterization of CBG genes (Underhill and Hammond, 1989) and studies of their expression in several mammalian species, as reviewed previously (Hammond, 1990). In terms of advancing our understanding of the biochemical properties of human CBG, knowledge of its primary structure provided a precise molecular size of 42,646 Da (Hammond et al., 1987). This had previously been difficult to determine biochemically because about 30% of the CBG mass is comprised of carbohydrates, which made the protein appear more like ~60 kDa when examined by size exclusion chromatography or denaturing polyacrylamide gel electrophoresis (Kato et al., 1988). The primary structures of CBGs in various mammalian species also revealed the locations of consensus sites for N-glycosylation, and allowed detailed studies of their utilization (Hammond et al., 1991; Avvakumov et al., 1993; Avvakumov and Hammond, 1994a; Avvakumov, 1995). Building on earlier biochemical studies of the oligosaccharides associated with human CBG (Avvakumov et al., 1993; Avvakumov and Hammond, 1994a; Avvakumov, 1995), these studies showed that CBG is only decorated by N-linked oligosaccharides, and that individual CBG molecules in biological samples vary considerably in the type and number of N-linked oligosaccharides that are associated with them, which provided an explanation for the substantial degree of electrophoretic micro-heterogeneity observed when CBG is examined (Mickelson et al., 1982; Robinson and Hammond, 1985; Kato et al., 1988; Nyberg et al., 1990; Ali and Bassett, 1995). Although the glycosylation of CBG obviously influences its half-life in the blood (Hossner and Billiar, 1981), which has been estimated to be about 5 days (Sandberg et al., 1964), the biological significance of the diversity in glycosylation remains obscure.

### 2.2. Steroid-binding site

The relative abundance in the blood, ease of purification and stability of CBG, when compared to other steroid-binding proteins or receptors, allowed its steroid-binding properties to be defined in great detail by Westphal (1986). These studies not only provided valuable information about the affinity binding constants for a comprehensive set of physiologically important steroid-ligands, which have been used extensively as a "benchmark data set" for the development of *in silico* modeling technologies (Cherkasov et al., 2006; Mager, 2006), but also identified critical functional groups on the steroid backbone that are prerequisites for affinity binding interactions, and paved the way for the design of various affinity labeling methods that provided early insight into the topography and location of the steroid-binding site (Marver et al., 1976; Khan and Rosner, 1977; Defaye et al., 1980). These studies predicted that a hydrophobic steroid-binding pocket contained cysteine, methionine, and histidine residues (Khan and Rosner, 1977; Defaye et al., 1980) and was likely located about 25 Å from the surface of the human CBG molecule (Defaye et al., 1980). Furthermore, early fluorescence quenching experiments (Marver et al., 1976) predicted that one of several tryptophan residues in human CBG was located in the steroid-binding site, and it was subsequently identified at position 371 in the mature human CBG sequence by steroid-affinity labeling (Grenot et al., 1994) and site-directed mutagenesis studies (Avvakumov and Hammond, 1994b). Based on the knowledge of the structural relationship between CBG and AAT/ACT, the crystal structures of which were well established (Baumann et al., 1991; Ryu et al., 1996), various predictions were made about where the steroid-binding site might be located (Edgar and Stein, 1995; Dey and Roychowdhury, 2003; Little and Rodriguez, 2005), but these remained speculative until the crystal structure of rat CBG was obtained in complex with cortisol (Klieber et al., 2007).

## 3. Crystal structures of CBG

### 3.1. Native steroid-bound state (stressed conformation)

The 1.93 Å resolution crystal structure of cortisol-bound rat CBG (Klieber et al., 2007) displays a typical serpin stressed (S) conformation, when adhering to the serpin structural nomenclature (Gettins, 2002). Native rat CBG contains a 5-stranded β-sheet A (termed s1A, s2A, s3A, s5A and s6A), a 6-stranded β-sheet B (s1B-s6B), a 4-stranded β-sheet C (s1C-s4C), and 10 intervening α-helices (hA-hJ) (Klieber et al., 2007). Furthermore, the closest structural relatives of rat CBG include clade A serpin members (Gettins, 2002) such as human AAT, ACT and TBG, and clade C and E members (Irving et al., 2000) such as antithrombin, and plasminogen activator inhibitor (PAI)-1 and -2 (Klieber et al., 2007). In addition, the RCL in the CBG steroid complex is fully exposed from the β-sheet A (Klieber et al., 2007), and this particular feature is shared by other serpins such as antithrombin (Jin et al., 1997) and heparin cofactor II (Baglin et al., 2002) which also have known ligand-binding sites, the occupancy of which causes their helix D to over-wind by two helical turns resulting in the concomitant expulsion of their RCLs (Fig. 1A).

Comparing the rat CBG (Klieber et al., 2007) and human TBG (Zhou et al., 2006) structures showed that their ligand-binding sites share similar features and are in exactly the same location within the serpin structure (Klieber et al., 2007). In the rat CBG crystal structure, 12 residues make close contact with cortisol through polar or hydrophobic interactions, at distances less than 4 Å (Klieber et al., 2007). As shown in Fig. 2, these residues include Q224 in s2B; T232 and F234 in s3B; F357 and K359 in s4B; W362 in s5B, R10, A13 and V17 in hA, and R252, I255 and D256 in hH. Moreover, in the

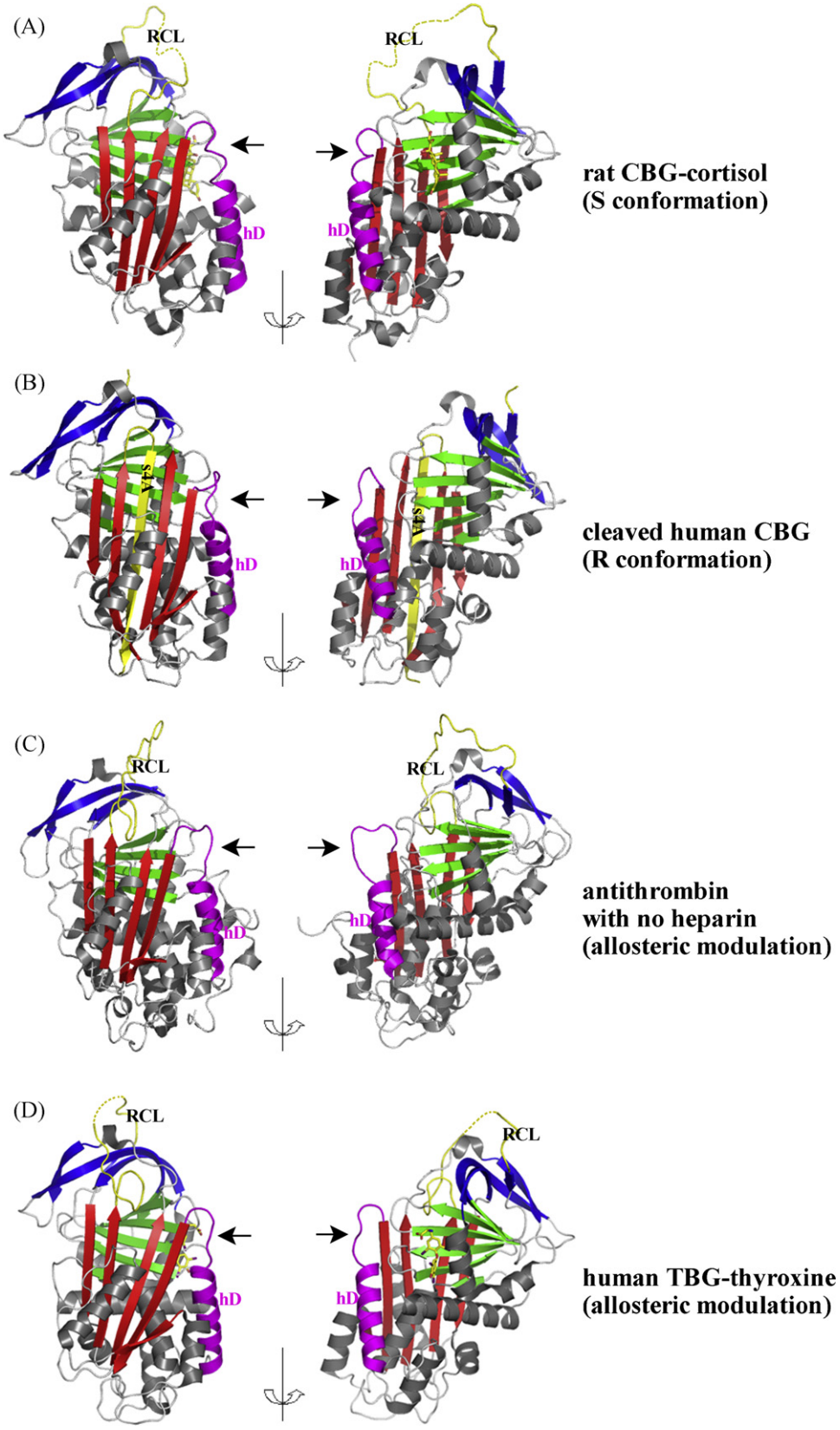


**Fig. 1.** Crystal structures of CBG, antithrombin and TBG: related serpins with ligand-binding properties. A, native rat CBG-cortisol structure (PDB code: 2v95). B, cleaved human CBG structure (PDB code: 2vdx). C, heparin-free antithrombin structure (PDB code: 1e05). D, human TBG structure bound to thyroxine (PDB code: 2ceo). The β-sheets A, B, and C are in red, green, and blue, respectively. Cleaved RCL fully inserted as a new β-sheet A (s4A) or intact RCL is coloured in yellow. Residues that could not be placed within the RCLs in rat CBG and human TBG structures are shown as dashed lines. Helix D (hD) is in magenta and other helixes are in grey. Cortisol and thyroxine are shown as stick models. The two-turn helical conformation on top of helix D and the partially unwinding of helix D are arrowed. The cartoons were prepared with program PYMOL (http://pymol.sourceforge.net). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

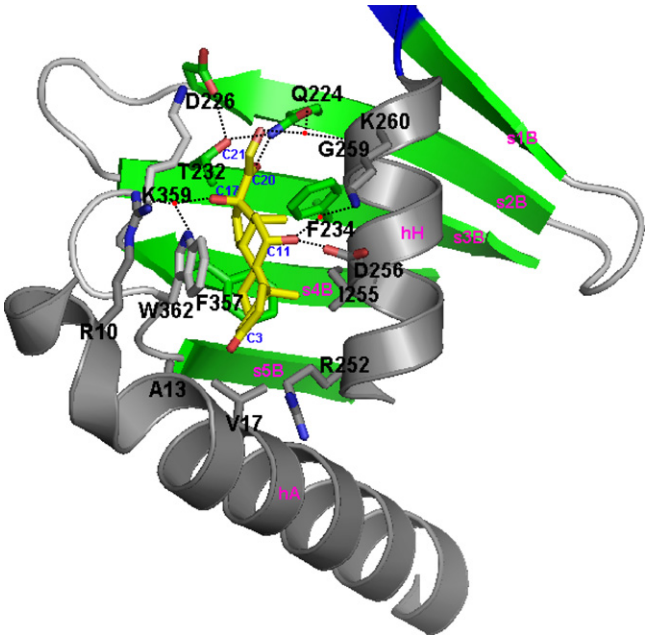


**Fig. 2.** Location of critical residues within the cortisol-binding pocket of rat CBG that participate in protein–ligand interactions. Side chains of residues interacting with cortisol are shown. Oxygen and nitrogen atoms are colored in red and blue, respectively. Water molecules are indicated as red dots. Polar interactions are represented by dotted lines. Other residues are involved in hydrophobic interactions (Klieber et al., 2007), which are not shown. hA, helix A. hH, helix H. Strands of β-sheet B are indicated as s1B, s2B, s3B, s4B, and s5B. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

steroid-binding pocket there are only three water molecules which bridge oxygen atoms from cortisol to G259 and K260 in hH, and Q224, K359 and W362 in β-sheet B (Klieber et al., 2007). Notably, in rat CBG the side chain of K359 points away from the steroid (Klieber et al., 2007), and this is relevant because in a cleaved human CBG-AAT chimaera structure this lysine residue is substituted by a histidine (H368), which potentially forms another hydrogen bond with cortisol (Zhou et al., 2008), and may account for preference of human CBG for cortisol versus corticosterone. It is therefore also likely that H368 is the histidine residue predicted previously to reside within the human CBG steroid-binding pocket (Defaye et al., 1980). Despite the predictions in the latter study that the human CBG steroid-binding site should also contain a methionine and cysteine, these residues were not observed in the CBG crystal structures. However, the side chains of the highly conserved cysteine 228 and methionine 364 in the human CBG-AAT chimaera structure point towards the binding site cavity (Zhou et al., 2008), and might be essential for packing of β-sheets. When compared to other serpin structures, including that of TBG, the rat CBG is unique in that there is a very obvious stacking interaction between the side chains of R10 in hA and W362 in s5B, and this plays a key role in positioning the highly conserved W362 within the rat CBG steroid-binding site so that it can make several points of contact with the steroid-ligand backbone (Klieber et al., 2007). Although the key role of this tryptophan residue (W371 in human CBG) was recognized from earlier affinity labeling and site-directed mutagenesis studies (Avvakumov and Hammond, 1994b; Klieber et al., 2007), the very important role played by the highly conserved R10 in rat CBG (R15 in human CBG) in anchoring this key tryptophan in position within the steroid-binding site was not appreciated prior to the resolution of the CBG crystal structure (Klieber et al., 2007).

### 3.2. Proteolytically cleaved state (relaxed conformation)

For most serpins in their native state, they adopt an S conformation in which the RCL is fully exposed for target proteinases to recognize. Upon proteolysis, the RCL is completely inserted into the central β-sheet A as a novel β-strand (s4A), resulting in a profound "stressed to relaxed" (S → R) conformational transition, and a concomitant increase in their thermo-stability (Irving et al., 2000). For human CBG, this occurs in concert with a decrease in steroid-binding affinity (Pemberton et al., 1988; Hammond et al., 1990a) following cleavage by neutrophil elastase.

The proteinase-cleaved human CBG crystal structure was solved at 1.84 Å resolution and at 2.30 Å resolution in a complex with cortisol (Zhou et al., 2008). In both structures, CBG underwent an irreversible S → R transition, with the full incorporation of cleaved RCL into β-sheet A, which was confirmed by a marked increase in thermo-stability upon RCL cleavage (Zhou et al., 2008). More strikingly, subsequent to the large movement of RCL, the β-sheet A is expanded, s2A is extended, and the top of helix D, which is in a helical conformation in native rat CBG (Klieber et al., 2007), unwinds and perturbs the configuration of cortisol-binding site (Zhou et al., 2008). As helix D runs along the rim of β-sheet A and contacts many residues in loop segments interconnecting strands of β-sheet B (Klieber et al., 2007), it is not surprising that it is a key element coupling inter-strand loop positioning and the steroid-binding site (Fig. 1B). However, it should be noted that these crystal structures of human CBG were obtained using an artificial human CBG containing a human AAT (Pittsburg) variant RCL sequence that is a target for thrombin cleavage (Zhou et al., 2008), and the registration of the cleaved RCL sequence as an s4A β-strand does not correspond to the length or sequence of an elastase-cleaved CBG RCL.

## 4. Allosteric mechanism of steroid binding and release

The ligand-bound antithrombin (Jin et al., 1997) and heparin cofactor II (Baglin et al., 2002) structures and the rat CBG-cortisol structure (Klieber et al., 2007) have several structural features in common: their RCLs are fully ejected, the β-sheet A is closed, and helix D is over-wound by two helical turns. Although we have not yet been able to obtain a ligand-free native CBG structure, the ligand-free (Mccoy et al., 2003) and cleaved (Mourey et al., 1993) antithrombin structures exhibit an RCL that is either partially inserted or fully incorporated into the central β-sheet, and this is accompanied by an unwinding of helix D. This observation prompted us to propose that an antithrombin-like allosteric coupling of RCL movement and steroid-binding site conformation might influence the steroid-binding properties of CBG (Klieber et al., 2007). In this model, complete insertion of the RCL coupled with the unwinding of helix D would irreversibly disrupt the CBG steroid-binding site, while partial insertion of RCL and the unwinding of helix D is reversible and would facilitate the equilibrated steroid binding and dissociation that is known to control the amounts of biologically active steroids under normal physiological conditions (Siiteri et al., 1982) (Fig. 1C). This type of allosteric modulation has also been suggested to influence the way TBG regulates the equilibrated binding and release of thyroxine by conformational changes, as illustrated in the crystal structure of thyroxine-bound human TBG, whose upper half of the central β-sheet is opened and reactive center loop is partially inserted to P14 threonine (Zhou et al., 2006) (Fig. 1D). Moreover, in the case of CBG it could also be of additional biological significance, because partially inserted RCL in unliganded CBG would be protected against protease cleavage, and this might ensure that unliganded CBG is protected to some extent from proteolysis (Klieber et al., 2007). However, this remains to be verified and may not be correct because

human CBG variants (W317F and W371K) that have very low affinities for steroid ligands, are not resistant to cleavage by neutrophil elastase attack, at least when studied *in vitro* (unpublished data).

The crystal structure of a thrombin-cleaved human CBG:AAT chimaera provided direct experimental evidence for the proposed allosteric coupling mechanism (Zhou et al., 2008). In this structure, incorporation of the cleaved RCL into the central β-sheet is indeed accompanied by a partial unwinding of helix D. A surprising finding was that despite this structural re-arrangement the steroid-binding site of the cleaved human CBG:AAT chimaera adopts a configuration that closely resembles that of the uncleaved CBG structure (Zhou et al., 2008). In addition, soaking of the crystals with cortisol partially restored occupation of the binding site. At present it is not clear whether or not this unexpected binding is caused by differences in the composition and length of the cleaved RCL in this structure in comparison to normal human CBG. It is also possible that the contacts between protein residues and steroid ligands within the binding site are highly sensitive to very minor structural perturbations. In this case, soaking the crystals in very high concentrations of cortisol, could have allowed for low affinity interactions to occur with cortisol even within a defective steroid-binding site. Although this might need further clarification, the comparison of the steroid-free and soaked cleaved CBG structures revealed in impressive detail how the occupation of the binding site allosterically modulates the conformation of helix D and how, upon reversal, unwinding of helix D is able to perturb the geometry of the distant cortisol-binding site.

## 5. Integrity of the CBG reactive center loop determines the stressed to relaxed transition

### 5.1. Reactive center loop (RCL) composition

When the CBG sequences from different mammalian species are aligned, the most poorly conserved region is the RCL. In the case of inhibitory serpins, the RCL acts as a bait for serine protease to interact covalently with (Hill and Hastie, 1987), and interspecies variability in this region (Hill and Hastie, 1987; Gettins, 2002) has been attributed to evolutionary pressures for serpins to adapt to species-specific environmental challenges (Hill and Hastie, 1987; Hammond et al., 1991). However, there is a general consensus in the RCL sequences of most serpins: P17 is always a glutamic acid, P16 glutamic acid/lysine/arginine, P15 usually glycine, P14 a non-charged, small residue mostly threonine or serine, and positions P12 to P9 are small residues, such as alanine, valine, serine, threonine, or glycine (Hopkins et al., 1993; Irving et al., 2000; Gettins, 2002). The highly conserved P17 glutamic acid appears to have an important stabilizing function (Irving et al., 2000), as illustrated by naturally occurring mutations at P17 in AAT (antitrypsin Z) (Stein and Carrell, 1995) and heparin cofactor II (Corral et al., 2004), and several other naturally occurring mutations within RCLs, such as at P10 of antithrombin III (Molho-Sabatier et al., 1989; Perry et al., 1989; Caso et al., 1991), as well as at P10 (Aulak et al., 1993), P12 (Skriver et al., 1991) and P14 (Davis et al., 1992) of C1-inhibitor, have all been linked to serpinopathies.

As illustrated in Table 1, analysis of the RCL sequences in CBGs from different species and in human TBG, ACT, antithrombin, heparin cofactor II, and AAT shows that the CBG RCLs display all the characteristics described above. There is a glycine residue at P15 except that CBGs in rodents have an asparagine at this position. However, the glycine at P16 in rodent CBGs substitutes for the P15 glycine in other CBGs, which was predicted by energy minimization and molecular dynamics calculations (Klieber et al., 2007) and re-affirmed by our mutagenesis study (Lin et al., 2009).

**Table 1**
Comparison of the reactive center loop sequences of CBGs in various mammalian species and the corresponding sequences of other related human serpins[a].

| Serpin | P17 | P16 | P15 | P14 | P13 | P12 | P11 | P10 | P9 | P8 | P7 | P6 | P5 | P4 | P3 | P2 | P1 | P1′ | P2′ | P3′ | P4′ | P5′ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Human CBG | E | E | G | V | D | T | A | G | S | T | G | V | T | L | N | L | T | S | K | P | I | I |
| Monkey CBG | E | E | G | V | N | T | T | G | S | T | G | V | T | L | N | P | M | S | K | P | I | I |
| Rat CBG | E | G | N | V | L | P | N | S | T | N | G | A | P | L | H | L | R | S | E | P | L | D |
| Mouse CBG | E | G | N | V | L | P | A | A | T | N | G | P | P | V | H | L | P | S | E | S | F | T |
| Rabbit CBG | E | H | G | G | V | E | V | A | A | T | G | G | P | L | Q | L | V | S | E | P | L | T |
| Sheep CBG | E | K | G | L | E | A | A | A | P | T | R | V | S | V | T | A | A | P | G | P | L | T |
| Pig CBG | E | K | G | M | E | A | A | A | P | T | T | R | G | R | S | L | H | A | A | P | K | P |
| TBG | E | K | G | T | E | A | A | A | V | P | E | V | E | L | S | D | Q | P | E | N | T | F |
| ACT | E | E | G | T | E | A | S | A | A | T | A | V | K | I | T | L | L | S | A | L | V | E |
| Antithrombin | E | E | G | S | E | A | A | A | S | T | A | V | V | I | A | G | R | S | L | N | P | N |
| Heparin cofactor II | E | E | G | T | Q | A | T | T | V | T | T | V | G | F | M | P | L | S | T | Q | V | R |
| AAT | E | K | G | T | E | A | A | G | A | M | F | L | E | A | I | P | M | S | I | P | P | E |

[a] The reactive center loop (RCL) sequences of CBGs from human (NP_001747), squirrel monkey (AAC60614), rat (NP_001009663), mouse (NP_031644), rabbit (P23775), sheep (CAA52000), and pig (NP_998977), and human TBG (NP_000345), and of human ACT (NP_001076), human antithrombin (NP_000479), human heparin cofactor II (NP_000176), and human AAT (NP_000286) are aligned. The numbering of residues in the RCL region is commonly in relation to the P1–P1′ (Met-Ser) proteinase cleavage site in human AAT (Gettins, 2002). The known protease cleavage sites are marked by grey boxes (Banda et al., 1987; Klieber et al., 2007). Macrophage elastase can attack AAT at P2–P1 and subsequently triggers its degradation (Banda et al., 1987). The cleavage site of macrophage elastase in oxidized human AAT is at P6–P7 (Banda et al., 1987), similar to that of neutrophil elastase in CBG at P5–P6 (Pemberton et al., 1988; Hammond et al., 1991).

In this context, our studies of the steroid-binding properties of human CBG variants with amino acid substitutions within the RCL region are of interest, because the cortisol-binding affinities of four variants, for example V336R at P14, in which the RCL appears to be constitutively expelled, are higher than those of wild-type CBG even before elastase cleavage (Lin et al., 2009). These intriguing observations support the concept that partial insertion of the intact RCL into the central β-sheet A exerts an allosteric effect on steroid binding of CBG in a normal physiological context, as described above, and raise the interesting possibility that natural CBG variants may exist with amino acid substitutions within the RCL region that have abnormally high steroid-binding affinities under normal conditions, or whose binding properties are not disrupted after proteolytic cleavage because of inefficient RCL insertion.

### *5.2. Key residues in the RCL of CBG that determine hormone release*

The P15–P9 portion of the RCL (defined as "the hinge") in serpins provides the essential mobility required for RCL insertion after proteolysis (Hopkins et al., 1993; Irving et al., 2000). In general, the kinetics of RCL insertion is sensitive to single amino acid substitutions (Gettins, 2002), and we have performed a detailed mutagenesis study in human CBG that has revealed crucial roles of residues at P15, P14, P12, P10 and P8 during insertion of the cleaved RCL (Lin et al., 2009). P15 glycine is highly conserved among all the serpins (Gettins, 2002); therefore it is not surprising that it plays an essential role in the type I β-turn in the repositioning of the cleaved RCL, and that the RCL of the G335P variant appears to remain expelled even after it is cleaved (Lin et al., 2009). Similarly, substitutions of the P14 Val to Arg and of the P12 Thr to Pro in human CBG also seem to block insertion of the cleaved RCL (Lin et al., 2009), supporting the important roles that P14 and P12 play in the RCL registration, and the leading role of P14 in this process is also supported by examples in other serpins (Hood et al., 1994; Lukacs et al., 1996; Huntington et al., 1997; Lawrence et al., 2000; Yamasaki et al., 2002). Interestingly, substitution of the P10 or P8 residues by a proline partially limits RCL insertion upon cleavage (Lin et al., 2009), and is in line with the conclusion drawn from the crystal structure of thyroxine-bound TBG that the P8 proline limits loop insertion within the upper half of the β-sheet A (Zhou et al., 2006).

## 6. Structural basis for genetic CBG variants

Several single nucleotide polymorphisms within the human CBG gene coding sequence that result in amino acid changes have been identified. To date, known hereditary forms of human CBG deficiency include transcortin Leuven (Leu93His variant) (Van Baelen et al., 1982,1993; Robinson and Hammond, 1985; Smith et al., 1992) and CBG Lyon (Asp367Asn variant) (Emptoz-Bonneton et al., 2000) that significantly reduce steroid-binding affinity, and a CBG null mutation caused by a frame shift within the codon for Trp12 in the secretion signal polypeptide sequence, which leads to the absence of CBG in plasma (Torpy et al., 2001). Most individuals heterozygous or homozygous for the CBG null allele or carriers of either the CBG Leuven or Lyon variants exhibit hypotension and/or fatigue. By contrast, although the common CBG Ser/Ala224 polymorphism does not influence steroid-binding activity (Smith et al., 1992), this polymorphism has been associated with chronic fatigue because it seems to be linked for some reason to low plasma CBG levels (Torpy et al., 2004). The CBG crystal structures have revealed how known naturally occurring genetic variants affect steroid-binding activities. In the human CBG structure model (Fig. 3A), Leu93 is located within helix D and close to the loop that connects strands s4B and s5B, which comprise the steroid-binding pocket. Asp367 is located at the rim of strand s4B and points towards Ser97 in helix D. Therefore, although Asp367 is not a key residue involved in steroid binding, it might participate in the allosteric coupling between the steroid-binding site and RCL insertion, as discussed above. By contrast, Ala224 is located within the loop connecting s1B and s2B, and its side chain points away from the protein and is far away from the steroid-binding pocket.

The naturally occurring rat CBG variant M276I (Smith and Hammond, 1991) and mouse CBG variant K201E (Lys200 in rat CBG) (Orava et al., 1994) also cause reductions in steroid-binding affinities. In the rat CBG structure (Fig. 3B), Met276 is displayed in s6A and its side chain points towards β-sheet B. Lys200 is a highly con-

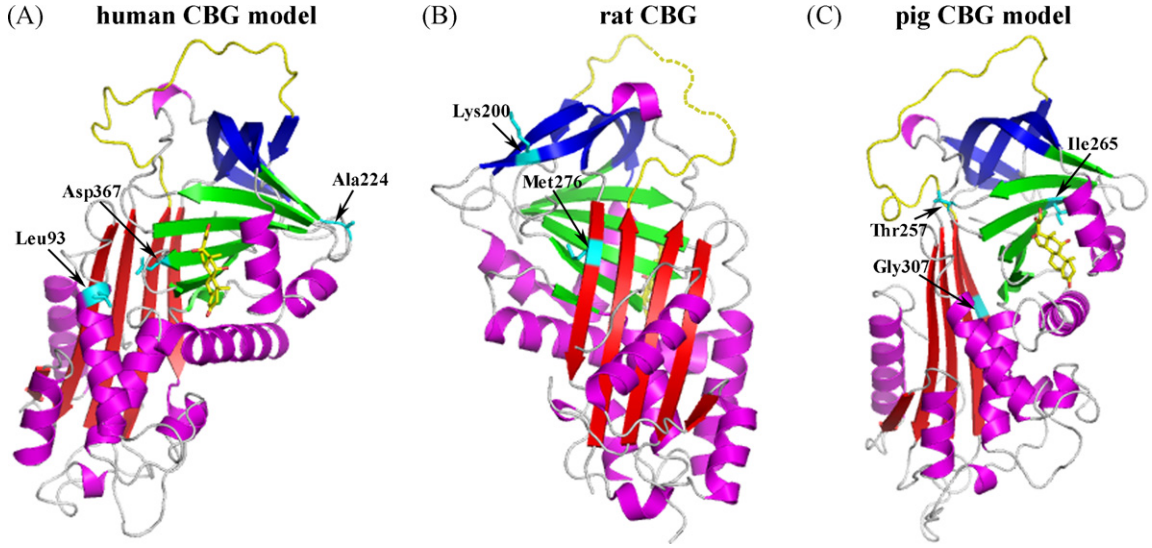


**Fig. 3.** Tertiary structures of human, rat and pig CBGs showing the positions of amino acid substitutions in naturally occurring CBG variants. A, human CBG; B, rat CBG (PDB code: 2v95); C, pig CBG. Models for CBGs from human and pig were developed using the SWISS-MODEL homology module (Arnold et al., 2006), with the structure of rat CBG (PDB code: 2v95) as a template. The β-sheets A, B, and C are in red, green, and blue, respectively. Helices are in magenta. RCL is coloured in yellow, and unlocatable residues of the RCL in rat CBG structure are shown as dashed lines. Cortisol is shown as stick model. Residues that are mutated in natural CBGs are coloured in cyan and their side chains are shown. The cartoons were prepared with PYMOL (http://pymol.sourceforge.net). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

served residue located in s2C, and its substitution may affect steroid binding by altering protein stability. The structural consequences have been discussed in detail in Klieber et al. (2007).

Recently an interesting study reported four pig CBG genetic variants, Arg307Gly, Ile265Val, Ser15Ile and Thr257Met, which are associated with abnormal cortisol levels and obesity in a pig intercross (Guyonnet-Duperat et al., 2006). It was found that Arg307Gly and Ile265Val mutations caused a decreased cortisol-binding affinity, whereas Ser15Ile, and Thr257Met did not appear to have any effects (Guyonnet-Duperat et al., 2006). Although homology modeling allows one to locate the corresponding residues with confidence within the general CBG fold (Fig. 3C), a structural rationale for the binding behavior of these mutants is not immediately obvious. Gly307 in pig CBG is conserved across mammalian species except for rodents, and is located within s6A. An arginine at this position could possibly favor a polar interaction with Asp61 located at the beginning of s6B, therefore stabilizing the overall fold and also indirectly the binding pocket. As a consequence the binding affinity could increase. Ile265 located in s3B and is part of a highly conserved stretch of amino acids. It does not directly contact the ligand but is part of the second or third shell of side chains surrounding the ligand-binding residues. A substitution at this position could therefore modulate ligand binding through a domino-like effect. Thr257 is displayed by the loop between s2B and s3B, is distant from the binding site and points away from the surface of CBG. This would be in agreement with the unaltered cortisol-binding affinity, when this residue is substituted by a methionine. The residue is however positioned close to helix D and therefore a substitution at this position could possibly affect the allosteric coupling mechanism that links the RCL insertion site to the steroid-binding position. Ser15 is located in the segment that precedes helix A, and equivalent residues are not visible in any of the solved CBG crystal structures. Therefore no structural insight can be derived through homology modeling for substitutions at this position.

## 7. Conclusions

Recent high-resolution crystallographic studies of CBG have provided a precise picture of its steroid-binding properties, and this information will be invaluable for the further refinement of *in silico* modeling methodologies that have relied on the CBG "benchmark data set" of steroid ligands. The CBG crystal structures have also revealed the mechanistic details of how CBG undergoes a typical serpin conformational transition after proteolytic cleavage of the RCL, and how this results in the irreversible release of its steroid ligands. While this is thought to be physiologically important at sites of inflammation where cleavage of CBG by neutrophil elastase may affect the targeted delivery of anti-inflammatory glucocorticoids, it may also be relevant in other physiological contexts because CBG is likely a substrate for other proteinases produced for instance at sites of tissue remodeling during development. Although the rat CBG crystal structure predicts an allosteric mechanism that would re-orient the RCL after occupancy of the steroid-binding site and cause a transition from a moderate to high-affinity steroid-binding state, this awaits further conformation through comparisons of the crystal structures of unliganded versus liganded native CBG molecules. The possibility that re-orientation of the RCL upon occupancy of the steroid-binding site also changes its access to specific proteinases could also be physiologically significant and warrants further investigation. Additional studies of how rodent CBGs are targeted and cleaved by proteinase are also needed and have been difficult to perform because the human neutrophil elastase that has been used to cleave human CBG does not recognize rat CBG. Insights into the structural and mechanistic requirements for RCL recognition by proteinases, and how the cleaved RCL inserts into the serpin R conformation, also suggest that possible polymorphism in the RCL sequence could influence ligand-binding affinity both prior to and after RCL cleavage (Lin et al., 2009). Moreover, the possibility that CBG variants exist in nature with abnormal RCL sequences has never been considered previously, and our recent *in vitro* studies of these types of possible human CBG variants suggest that the consequences of improper or incomplete insertion of the RCL sequence as an s4A β-strand, could result in CBG molecules with a constitutively active steroid-binding site even after the RCL is cleaved by a proteinase (Lin et al., 2009).

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