Pergamon

0960-0760(95)00099-2

J. Steroid Biochem. Molec. Biol. Vol. 53, No. 1–6, pp. 515–522, 1995

0960-0760/95 $9.50 + 0.00

Structure and Function of Corticosteroid-binding Globulin: Role of Carbohydrates

George V. Avvakumov

MRC Group in Fetal and Neonatal Health and Development, University of Western Ontario and London Regional Cancer Centre, and Victoria Hospital Research Institute, London, Ontario, Canada

To study the site-specificity of human corticosteroid-binding globulin (CBG) glycosylation and the functional significance of individual carbohydrate chains in its molecule, a panel of recombinant CBG mutants containing each of the six potential glycosylation sites alone and in various combinations has been expressed in Chinese hamster ovary (CHO) cells. Analyses of these mutant glycoproteins showed that three of the glycosylation sites are only partially utilized, and this may contribute to the production of glycoforms with distinct physiological functions. Processing of individual carbohydrate chains (branching and fucosylation) is site-specific and may, thus, account for the formation of structural determinants essential for the recognition of CBG by cell membranes. Glycosylation at the only phylogenetically conserved consensus site, Asn^{238}–Gly^{239}–Thr^{240}, is essential for the biosynthesis of CBG with steroid-binding activity. Evidence has been obtained to support the hypothesis that transient carbohydrate–polypeptide interactions between Trp^{266} and the maturing carbohydrate chain at Asn^{238} occur during early stages of the CBG biosynthesis which affect protein folding and formation of the steroid-binding site. Another tryptophan residue, Trp^{371}, has been found to be critical for CBG–steroid interactions and is likely located in the steroid-binding site.

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INTRODUCTION

Glycosylation is a common type of protein modification in eukaryotes which provides proteins with an additional level of structural complexity and affects their biosynthesis, secretion and biological activity [1, 2]. Although removal of carbohydrates from some glycoproteins does not influence their biochemical activity, it is generally recognized that the complete function of a glycoprotein within complex organism is often intimately linked to the structure of its carbohydrate component [1]. Variations in the carbohydrate portions of glycoproteins are often associated with alterations in physiological state and may result in the appearance of glycoforms with modified physiological activity [3]. Studies of multi-glycosylated proteins have shown that oligosaccharide chains attached to the same molecule usually differ in their structure and impact on the formation and stabilization of an active molecular conformation [4]. However, the need for specific carbohydrate structures at particular locations is as yet poorly defined with respect to both biosynthesis and function of glycoproteins [5].

Corticosteroid-binding globulin (CBG) represents a useful model for studying how differential glycosylation may influence the biosynthesis and biological activity of an extracellular glycoprotein. Plasma CBG in vertebrate species invariably contains 20–30% carbohydrate [6], the composition of which is species-specific [7, 8] and can alter during fetal development [9], pregnancy [10, 11] and some diseases [12]. In particular, CBG derived from human donor serum contains about 5 moles of *N*-glycosidically linked biantennary and triantennary (in a molar ratio of 3:2) oligosaccharides of the *N*-acetyllactosamine type per 1 mole of glycoprotein [13]. However, there are six consensus sites for *N*-glycosylation in the amino-acid sequence of human CBG [14]. Previous studies of the impact of carbohydrates on the steroid-binding activity of CBG have produced conflicting results [15, 16]. It is known, however, that carbohydrates regulate the blood level of CBG by influencing its plasma half-life [17] and may affect its compartmentalization within the organism [18]. More importantly, carbohydrate structures on the CBG molecule are essential for the interaction

Proceedings of the IX International Congress on Hormonal Steroids, Dallas, Texas, U.S.A., 24–29 September 1994.

between CBG–steroid complexes and the plasma membranes of specific target cells, which seems to be a crucial step in the guided delivery of steroid hormones into tissues [19]. To explore the processing and biological significance of individual carbohydrate chains in human CBG, a series of recombinant CBG mutants have been prepared containing each of the six potential glycosylation sites alone or in various combinations, and their molecular properties are reviewed here in relation to structural and functional significance of individual oligosaccharide chains present in CBG.

EXPERIMENTAL

Construction and expression of CBG mutants

A cDNA for the human CBG precursor [14] was inserted into a *Hin*dIII/*Xba*I-digested pSelect-1 phagemid (Promega, Madison, WI) and mutated to convert the codons for Asn (AAC or AAT) within consensus sites for *N*-glycosylation to those for Gln (CAA or CAG, respectively), according to the protocol recommended by Promega. When this resulted in mutants lacking steroid-binding activity, site-directed mutagenesis was also performed to disrupt the appropriate consensus sites (Asn–Xaa–Thr) in an alternative manner by substitution of Thr^{76} or Thr^{240} (ACT) with Ala (CGT). Oligonucleotides complementary to the sense strand of the cDNA and containing one or two altered nucleotides in the central part of their sequence were synthesized by the Molecular Biology Core Facility of the Medical Research Council Group in Fetal and Neonatal Health and Development (London, Ontario) and were used as mutagenesis primers. The CBG glycosylation sites were mutated individually, and cDNAs containing multiple mutations were constructed by combining appropriate portions of individually mutated cDNAs. Separate mutations were performed to convert individually each of the four codons for Trp (TGG) in cDNAs, encoding either wild-type CBG precursor or the glycoprotein containing only one glycosylation site at position 238, to a Phe codon (TTT). The Trp codons at positions 141 and 371 were also converted into ACG (Thr) and AAG (Lys), respectively. The mutated cDNAs were sequenced [20] to confirm that only targeted mutations had occurred and then were subcloned into a *Hin*dIII/*Xba*I-digested pRc/CMV vector (Invitrogen, San Diego, CA) for the expression in Chinese hamster ovary (CHO, pro^-, wild-type) cells [21]. After selection in the presence of Geneticin (Gibco/BRL, Burlington, Ontario), stably-transformed cells were expanded to near confluence, washed twice with PBS, and then cultured for 2 days in Dulbecco's modified Eagle medium containing 100 nM cortisol.

Binding assay and immunochemical analysis

The steroid-binding capacity of recombinant proteins in culture medium was determined by saturation assay using [^{3}H]cortisol as labelled ligand [22], and their affinity for cortisol was measured by Scatchard analysis [23]. Their immunochemical properties were examined by radioimmunoassay (RIA) using a rabbit anti-human CBG antiserum and ^{125}I-labelled [24] human CBG that was purified as described previously [25]. Appropriate dilutions of a human serum sample with a known concentration of CBG were used to construct the calibration curve.

Western blotting

Polyacrylamide gel electrophoresis (4% stacking gel and 10% resolving gel) in the presence of SDS (SDS–PAGE) was performed as previously described [26], and proteins were transferred to a Hybond-ECL (Amersham, Oakville, Ontario) membrane by electroblotting [27] at 300 mA for 1 h. The blots were blocked and incubated with an anti-human CBG antiserum (1:500 diluted), and immunoreactive proteins were visualized using the ECL Western blotting analysis system (Amersham).

Lectin-affinity chromatography

For chromatography on immobilized concanavalin A (Con A), samples (0.5–1 ml) of culture medium were adjusted by ultrafiltration to contain similar concentrations (4–5 nM) of immunoreactive CBG and applied onto 3 ml Con A-Sepharose (Pharmacia, Baie d'Urfé, Quebec) column pre-equilibrated in lectin column buffer (50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, containing 0.01% bovine serum albumin), followed by 0.5 ml of this buffer. After a 15 min delay to allow glycoproteins to interact with the affinity gel, elution was performed with 9 ml of the lectin column buffer followed by 15 ml of the same buffer containing 20 mM methyl-α-D-mannopyranoside. This procedure resulted in complete elution of the glycoproteins from the column: subsequent washing with 15 ml of the buffer containing 200 mM mannoside gave no additional immunoreactive material in the eluate. Fractions of 1 ml were collected. Chromatography on immobilized lectin from *Lens culinaris* (LCA) was performed essentially as above using a 0.5 ml column packed with LCA covalently attached to 4% beaded agarose (Sigma, St Louis, MO). The sample volume was 0.5 ml, and two 1.5 ml fractions were collected: the first was eluted with the lectin column buffer and the second with the same buffer containing 100 mM methyl-α-D-mannopyranoside. In both cases, the chromatographic fractions were analyzed by RIA, and the amount of CBG recovered from the column after elution with the buffer containing mannoside (Con A+ or LCA+, respectively) was expressed as a percentage of the total amount of CBG in all chromatographic fractions.

Enzymatic desialylation and deglycosylation

Culture media were concentrated by ultrafiltration to yield a CBG concentration of 4–6 nM. During this procedure, the buffer was changed to 0.1 M sodium phosphate, pH 7.2, containing 25 mM EDTA and 0.5 μM cortisol to stabilize the proteins. The samples were digested for 16 h at 37°C with neuraminidase (EC 3.2.1.18) from *Clostridium perfringes* (Boehringer Mannheim, Laval, Quebec). For desialylation or complete removal of the carbohydrates from the mutant with a single glycosylation site at position 238, digestion was performed for 72 h at 37°C with neuraminidase alone or with a combination of neuraminidase and recombinant *N*-glycosidase F (EC 3.5.1.52, Boehringer Mannheim), respectively. Reaction products were analyzed by SDS–PAGE/Western blotting, and their parameters of steroid binding were determined as described above.

RESULTS AND DISCUSSION

Site-specificity of CBG glycosylation

Table 1 illustrates the location of consensus sites for *N*-glycosylation in the wild-type and mutant forms of human CBG expressed in CHO cells, together with the concentrations of immunoreactive CBG in culture media. In a standard RIA that employed a polyclonal anti-CBG antiserum, dose–response curves for all CBG mutants were similar to those obtained for wild-type recombinant or serum-derived CBG [21, 28]. This indicates that site-directed mutagenesis did not alter the overall conformation of CBG and validated the use of RIA for the measurement of concentrations of the mutants. In general, production of the mutants was lower than that of the wild-type glycoprotein and was markedly decreased when all six consensus sites were eliminated. However, carbohydrates attached to different glycosylation sites appear to have different significance for the CBG biosynthesis and secretion, sites II and IV being most important. Removal of each one of them resulted in substantial decrease in the CBG concentration in culture medium, while the mutants having only a single site II or IV were secreted at levels close to that of wild-type CBG.

Western blotting of the six mutants each of which lacks one of the glycosylation sites showed that all of them had a higher electrophoretic mobility than the wild-type glycoprotein, thus indicating that all the sites may be utilized in the course of CBG biosynthesis [21]. However, electrophoretic properties of these mutants, as well as other mutants with an equal number of the glycosylation sites, were found to be different [21], and this was the first indication that the processing of the CBG oligosaccharide chains may be site-specific. To explore this issue, mutants containing only one glycosylation site were analyzed by Western blotting in combination with lectin-affinity chromatography [28].

Table 1. Location of N-glycosylation sites in CBG mutants and their relative concentrations in culture medium

Location of consensus sites for *N*-glycosylation in wild-type (# 1) and mutant proteins (# 2– # 21)	Relative amount (%) in culture medium
1. I II III IV V VI N–NMS NLT NKT NGT NFS NLT–C	100
2. N–QMS–C	74±15
3. N–QLT–C	41±10
4. N–NLA–C	69±12
5. N–QKT–C	85±2
6. N–QGT–C	52±15
7. N–NGA–C	45±3
8. N–QFS–C	113±18
9. N–QLT–C	79±12
10. N–QLT QGT–C	58±6
11. N–NLA NGA–C	52±7
12. N–NLA QKT QGT QFS QLT–C	8±1
13. N–QMS QKT QGT QFS QLT–C	74±7
14. N–QMS NGT NFS NLT–C	75±7
15. N–QMS NLA QGT QFS QLT–C	9±1
16. N–QMS NLA QKT QFS QLT–C	78±2
17. N–QMS QKT QFS QLT–C	74±12
18. N–QMS NLA QKT QGT QLT–C	11±3
19. N–QMS NLA QKT QGT–C	42±10
20. N–QMS NLA QKT QGT QFS–C	9±4
21. N–QMS QLT QKT QGT QFS QLT–C	5±2

Relative positions and sequences of consensus sites for *N*-glycosylation (I–VI) are shown for wild-type CBG. Only the sequences of altered consensus sites are shown for mutated proteins. The amounts of CBG in culture media from transfected CHO cells were assayed by RIA and expressed as a percentage of secreted wild-type CBG (average values ± SE of three determinations).

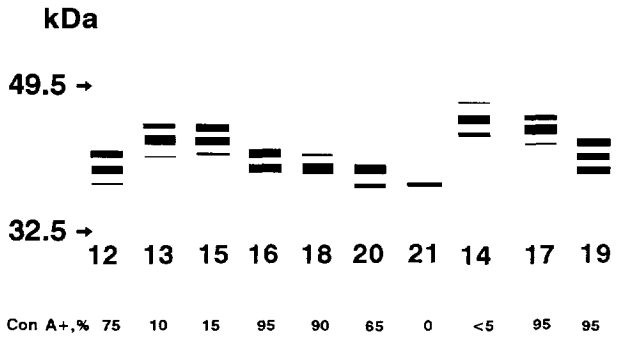

Fig. 1. Diagram of Western blots of CBG mutants containing one or two glycosylation sites (as indicated below the lanes) and unglycosylated CBG (mutant 0). Relative amounts (Con A+, %) of material retarded in the column and eluted with 20 mM methyl-α-mannopyranoside during Con A chromatography of the corresponding mutants are indicated below the lanes. Positions of pre-stained molecular weight (kDa) marker proteins are shown by arrows.

Differences in their electrophoretic mobilities (Fig. 1) reflect variations in the utilization of glycosylation sites and differential processing of the carbohydrate chains. An immunoreactive band corresponding to unglycosylated CBG is visible in the samples of the mutants containing the glycosylation site closest to the N-terminus of the CBG polypeptide or one of the two glycosylation sites located near the C-terminus. Such partial utilization of the consensus sites closest to the ends of the polypeptide chain has been reported for other glycoproteins [29] and may result in the formation of functionally dissimilar glycoforms of CBG.

Results of Con A-chromatography, along with electrophoretic patterns of mutants containing two glycosylation sites, (Fig. 1) led us to the conclusion that biantennary oligosaccharides of the *N*-acetyllactosamine type are preferentially attached to glycosylation sites I, IV, V and VI, whereas more branched (more likely triantennary, as in serum-derived CBG) sugar chains are present at sites II and III. Affinity

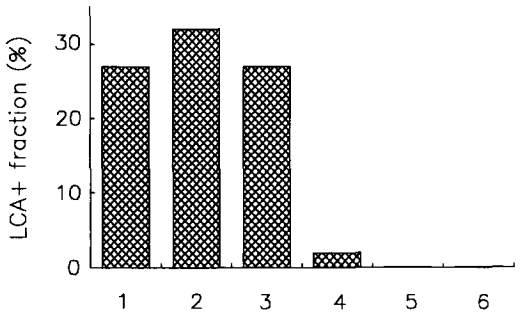

Fig. 2. LCA-column chromatography of CBG purified from human serum (1), recombinant wild-type CBG (2), and mutants containing only one biantennary chain attached to glycosylation site I (3), IV (4), V (5) or VI (6). Relative amounts of proteins retarded by the column are expressed as a percentage of the total immunoreactive CBG eluted from the column.

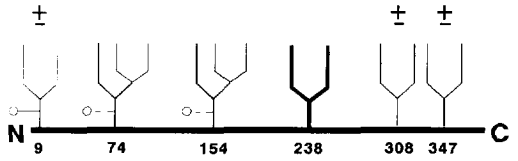

Fig. 3. Glycosylation of human CBG [28]. Preferential locations of biantennary and triantennary chains at Asn residues indicated by numbers are shown. Partial glycosylation of sites I, V and VI is shown by the symbol ±. Fucose residues are shown by circles, with dashed lines indicating putative attachment of these monosaccharides to one or both triantennary chains. The carbohydrate chain which is essential for steroid-binding activity of CBG is shown in bold.

chromatography on immobilized LCA (Fig. 2) indicated that only one of the three biantennary chains, namely, one attached to site I, contains a significant amount of fucose. Attachment of this monosaccharide to the sugar chains of the *N*-acetyllactosamine type may significantly alter their conformation [4] and, consequently, potential as determinants for molecular recognition. It is important that the chromatographic behaviour of natural CBG on the LCA-agarose column was similar to that of the mutant having only oligosaccharide chain at site I (Fig. 2). This further confirms that glycosylation of recombinant CBG is similar, even in fine details, to that of its natural counterpart and suggests that majority of the fucose present in serum-derived CBG (total content is 1.2 mol of fucose/mol of glycoprotein) is located in either one or both more processed oligosaccharide chains attached to sites II and III. (Fucose residues attached to triantennary chains are inaccessible for the lectin used in this study, and localization of this monosaccharide to one or both triantennary chains must await further investigation). The results of this part of the study are summarized in Fig. 3.

Glycosylation and steroid-binding activity of CBG

When the concentrations of immunoreactive CBG in culture media were compared to the corresponding binding capacity measurements (Fig. 4), it was apparent that removal of glycosylation sites I, III, V, and VI had little or no effect on the CBG binding activity. Mutation of Asn^{74} (site II; point 3 in Fig. 4) resulted in a significant decrease in the cortisol-binding ability, but an alternative mutation of site II, substitution of Thr^{76} with Ala, resulted in a mutant (point 4, Fig. 4) with a binding capacity that closely correlated with its concentration measured by RIA. By contrast, elimination of site IV by substitution of either Asn^{238} with Gln or Thr^{240} with Ala (points 6 and 7, respectively, Fig. 4) resulted in a complete loss of the steroid-binding activity. On the contrary, a mutant containing only glycosylation site IV was clearly able to bind cortisol ($K_a = 1.1 \times 10^8\ M^{-1}$). These data suggest that the carbohydrate chain attached to Asn^{238}, the only glycosylation site which is phylogenetically conserved

(Fig. 5), is essential for the formation of the CBG steroid-binding site.

Intramolecular carbohydrate–protein interactions

The importance of the oligosaccharide attached to glycosylation site IV for the steroid-binding activity of CBG implies one of the following three possibilities: (i) carbohydrates at this position actively participates in steroid binding; (ii) they are essential for the conformational stability of the protein; or (iii) they only affect the folding of the nascent polypeptide that leads to the formation of the steroid-binding site. The first possibility is unlikely because almost complete removal of carbohydrates from human CBG had no influence on its affinity for cortisol [15]. The latter two possibilities both imply that an intramolecular carbohydrate–polypeptide interaction occurs, and the limited processing of the oligosaccharide attached to site IV (minimal branching and no fucose residues) supports this assumption. To distinguish between the two possibilities we assessed the consequences of enzymatic deglycosylation of a CBG mutant, containing only this particular oligosaccharide chain, with respect to its steroid-binding properties [28]. Treatment of this mutant with a mixture of neuraminidase and *N*-glycosidase F under non-denaturing conditions resulted in the production of totally deglycosylated CBG with the affinity for cortisol equal to those of untreated glycoprotein. Thus, it appears that the carbohydrate chain attached to Asn^{238} is necessary for the CBG molecule to acquire its active conformation. Once this has occurred, it no longer influences the conformation of the steroid-binding site, which must therefore be maintained by the overall tertiary structure of the polypeptide. However, the conformation stability of CBG depends on the presence of carbohydrates: after incubation for 72 h at 37°C, the binding capacity of the deglycosylated protein was approximately two times lower than that of the control sample that was similarly incubated in the absence of glycosidases [28].

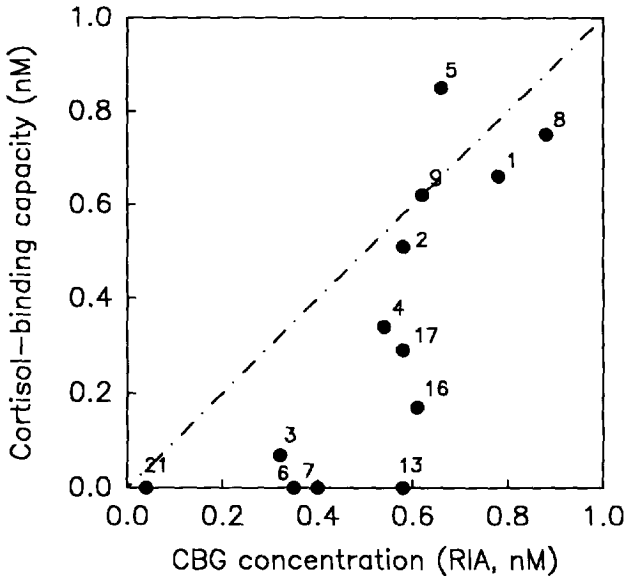

Fig. 4. Concentrations of immunoreactive CBG and cortisol-binding capacity of the recombinant proteins in culture medium. Data points correspond to the proteins as numbered in Table 1. The dotted line represents an ideal case when concentration of the immunoreactive protein is exactly the same as its binding capacity.

The primary structure of human CBG is closely related to that of α_1-proteinase inhibitor [14]. It has been supposed [30] that the carbohydrate chain attached to Asn^{247} in the latter glycoprotein (position analogous to Asn^{238} in CBG) interacts with a tryptophan residue. This amino acid has also been found in the active sites of carbohydrate-transforming enzymes and lectins [31, 32], and we therefore focused our attention on the tryptophan residues in CBG as candidates for the intramolecular carbohydrate–protein interactions. Since there are four tryptophan residues in human CBG (Fig. 5), we employed site-directed mutagenesis in order to individually substitute them with residues of another hydrophobic amino acid, phenylalanine, in the CBG mutant containing only glycosylation site IV. Mutations at positions 141, 185 and 371 resulted in products with electrophoretic mobilities and lectin-binding properties similar to those of the parent glycoprotein, whereas mutation of Trp^{266} completely abolished secretion of the glycoprotein [28]. When similar mutations were performed in CBG containing all six glycosylation sites, only one of the mutations, involving Trp^{266}, resulted in the production of a glycoprotein, showing certain affinity for cortisol, with higher molecular mass than that of wild-type CBG [33], and this is probably due to a difference in glycosylation, i.e. more complete utilization of the consensus sites and/or deeper processing of the oligosaccharide chains. Taken together, the above data suggest that an interaction between carbohydrates attached to Asn^{238} and Trp^{266} contributes to the proper folding of the nascent CBG molecule, but the presence of the other carbohydrate chains makes possible other interactions within the CBG molecule which may partially compensate for the absence of Trp^{266}. Furthermore, Trp^{266} is obviously only one of the amino acid residues involved in the intramolecular carbohydrate–protein interactions. We can also assume that monosaccharide units belonging to the "core" region of the essential carbohydrate chain are more important that those forming the "outer antennae". This would explain normal steroid-binding activity of pregnancy-associated CBG variant [10], that contains only triantennary oligosaccharides, and recombinant CBG produced in yeast cells *Pichia pastoris* which can synthesize only less processed, high-mannose oligosaccharides (our unpublished data). Besides, this assumption is in line with the known pathways of the processing of glycoprotein oligosaccharides which accompany folding of newly synthesized molecules in endoplasmic reticulum [1].

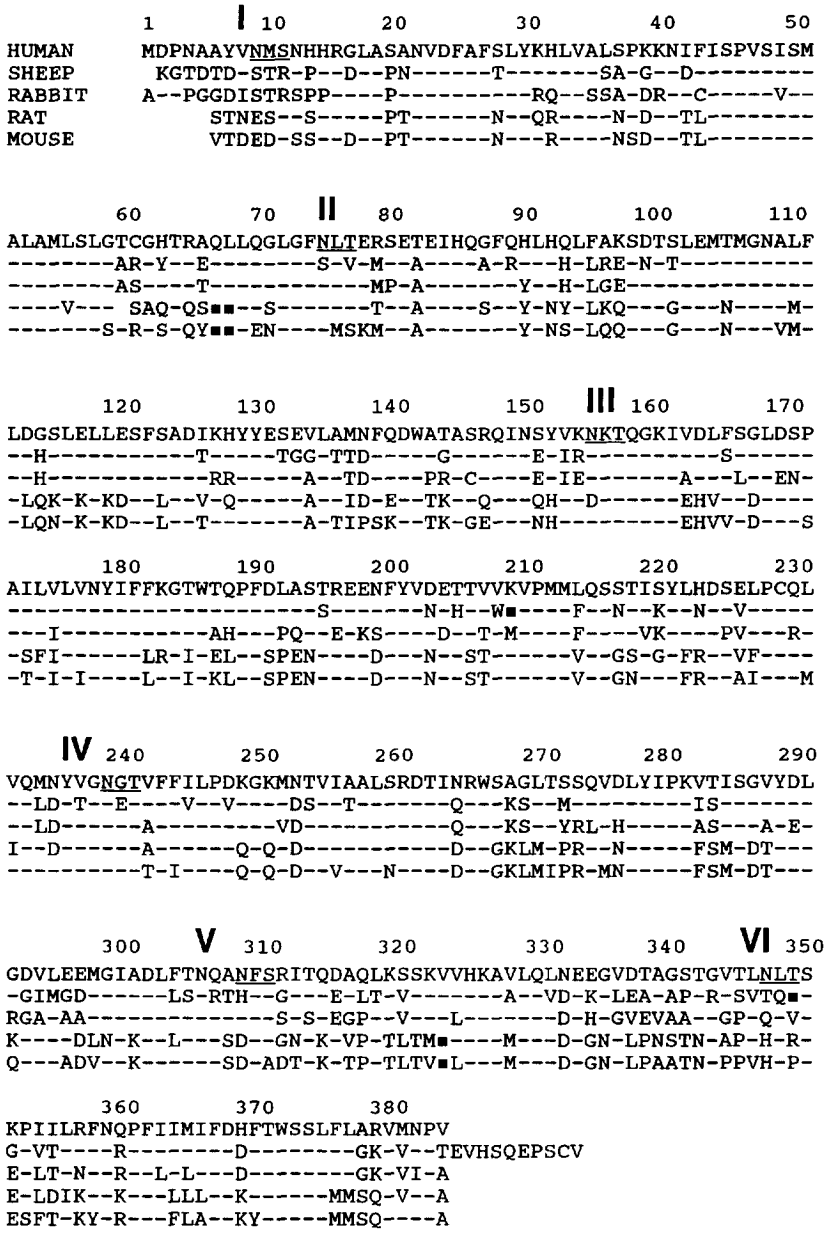

Fig. 5. Phylogenetic comparison of cDNA-deduced amino-acid sequences of CBG from human [14], sheep [9], rabbit [11], rat [37] and mouse [38]. Numbering of amino-acid residues and glycosylation sites (underlined, Roman numerals) corresponds to the primary structure of human CBG.

The tertiary structure of CBG and the topology of its steroid-binding site remain to be determined. However, earlier experiments indicated that one of the four tryptophan residues present in human CBG is important for the steroid binding, probably, due to a strong dipole–dipole interaction between its indole group and ring A of a 4-ene-3-ketosteroid molecule [34]. There are four tryptophan residues in human CBG and all of them are conserved phylogenetically (see Fig. 5). Comparison of CBG with other members of the serpin superfamily [35] shows that Trp^{185} is invariably conserved in all of them and either tryptophan or phenylalanine is present in a position analogous to Trp^{266} in human CBG. Consequently, these two residues are likely to be important for the general conformation of serpins. In accordance with this, substitution of these residues with phenylalanine, an amino acid that is not capable of the kind of dipole–dipole interactions mentioned above, did not abolish high-affinity binding of cortisol (Table 2). Substitution of Trp^{141} with phenylalanine also preserved the CBG ability to bind steroids although with a lower affinity. However, when this tryptophan residue was substituted with threonine, the residue located in an analogous position in the archetype of the serpin superfamily, α_1-proteinase inhibitor [35], the binding activity was completely eliminated (Table 2). This suggests that hydrophobic interactions between

Table 2. Effect of mutation of individual amino acid residues on the secretion and steroid-binding activity of human CBG expressed in CHO cells

	Secretion		Affinity for cortisol	
Mutation	nM	%*	$K_a \times 10^{-8}$	%*
No (wild-type)	1.2†	100	9.2†	100
Y29F	1.5	125	7.0	76
Y29P	ND‡	—	ND	—
S44A	1.3	108	6.9	75
S47A	0.9	75	6.0	65
W141F	0.9	75	0.9	10
W141T	0.35	29	ND	—
W185F	0.35	29	1.8	20
Q232L	1.7	142	ND	—
W266F	0.55	46	1.2	13
S267G	1.0	83	0.5	5
S267L	0.8	67	0.5	5
M364W	1.1	92	ND	—
M364C	1.1	92	8.3	90
F366I	0.9	75	2.4	26
W371F	1.2	100	ND	—
W371K	1.1	92	ND	—

Data are means of 2 determinations unless otherwise indicated.
*Corresponding values for wild-type CBG were taken as 100%.
†Mean values from eight experiments.
‡ND, not detectable.

the residue at position 141 and the steroid ligand or other amino acids in the CBG polypeptide is important for the steroid binding. Mutations of Trp^{371} invariably resulted in the total lack of the CBG ability to bind steroids (Table 2), although the mutant proteins were secreted at a level similar to that of wild-type CBG which argues against significant impairment of the overall protein conformation. We, therefore, conclude that Trp^{371} is located in the steroid-binding site and directly involved in the interaction with steroid ligands. This conclusion has been recently confirmed by the result of an independent study by Grenot *et al.* [36] who found that photolabeling of CBG with Δ^6-derivatives of cortisol, corticosterone and progesterone resulted in a covalent modification of Trp^{371}. Table 2 also illustrates effects of substitutions of several other amino-acid residues on the secretion and binding activity of CBG. These data allow exclusion of some of them from consideration as candidates for the residues participating in the formation of the steroid-binding site (notably, those located in the N-terminal portion of CBG polypeptide) and indicate the importance of Gln^{232} and Ser^{267} for biological activity of CBG. The fact that the substitution of Met^{364} with a bulky tryptophan residue, but not with chemically related cysteine, compromises the steroid-binding activity of CBG indicates spatial constrains in the C-terminal part of CBG around this particular residue. It is noteworthy that a mutation involving Phe^{366} had only a slight effect on CBG binding activity. These data may be helpful while modelling the three-dimensional structure of CBG. I believe, however, that until experimental data on the tertiary structure of CBG are available, site-directed mutagenesis can be more effectively utilized for the preparation of proteins with "calculated" mutations which would involve amino-acid residues that are invariably conserved in the primary structures of CBG from various species but not in other members of serpin superfamily. Our preliminary analysis has shown that there are only a few such "CBG-specific" residues, and experiments on their modification are currently under way in this laboratory.

Acknowledgements—This work was performed in the laboratory of Dr Geoffrey Hammond at The London Regional Cancer Centre and was supported by the Medical Research Council of Canada, Archangelo Rea Foundation and Allelix Biopharmaceuticals Inc. The technical assistance of Susan Warmels-Rodenhiser, Allen Grolla and David Dales is gratefully acknowledged. I also thank Gail Howard for her excellent secretarial help.

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