Pergamon

0960-0760(94)E0033-H

J. Steroid Biochem. Molec. Biol. Vol. 49, No. 2/3, pp. 191–194, 1994

0960-0760/94 $7.00 + 0.00

Substitutions of Tryptophan Residues in Human Corticosteroid-binding Globulin: Impact on Steroid Binding and Glycosylation

George V. Avvakumov* and Geoffrey L. Hammond

Departments of Obstetrics and Gynaecology, Biochemistry, and Oncology, and MRC Group in Fetal and Neonatal Health and Development, University of Western Ontario, London, Ontario, Canada

Human corticosteroid-binding globulin (CBG) contains four tryptophan residues at positions 141, 185, 266 and 371; one of which is thought to be located in the steroid-binding site. These residues were substituted by site-directed mutagenesis and expression of mutant CBG cDNAs in Chinese hamster ovary cells. Analyses of the resulting mutants indicate that Trp^{371} is most likely located in the steroid-binding site, and that hydrophobic interactions between Trp^{141} and the steroid molecule or other amino-acids in the CBG polypeptide may also contribute to high-affinity interactions between CBG and its steroid ligands. In addition, substitution of Trp^{266} resulted in altered glycosylation of CBG, and this supports the concept that it participates in intra-molecular carbohydrate–polypeptide interactions which may influence the conformation and secretion of this glycoprotein.

J. Steroid Biochem. Molec. Biol., Vol. 49, No. 2/3, pp. 191–194, 1994

INTRODUCTION

Corticosteroid-binding globulin (CBG) is a plasma glycoprotein that binds cortisol and progesterone and modulates their bioavailability [1–3]. The tertiary structure of CBG remains to be determined, but experiments using affinity-labelling and various spectroscopic techniques have provided information about the nature of amino acids within the steroid-binding site, and its general topology [4–7]. The importance of a tryptophan for the high affinity-binding of Δ^4-3-keto-steroids to human CBG has been indicated by fluorescence quenching [8, 9], affinity-labelling [10], and ultraviolet difference spectroscopy [11]. This suggests that a tryptophan indole group is oriented in close proximity to ring A of steroid ligands to allow a strong dipole–dipole interaction between them [11]. If this is true, substitution of this tryptophan with any other amino acid should abolish the steroid-binding activity of CBG.

Human CBG contains six potential sites for *N*-glycosylation that are at least partially utilized when the protein is expressed in Chinese hamster ovary (CHO) cells [12]. Attachment of a biantennary complex-type oligosaccharide to Asn^{238} is absolutely necessary for the creation of the steroid-binding site [12], and we have recently demonstrated that this chain probably interacts with a tryptophan residue [13].

There are four tryptophan residues at positions 141, 185, 266 and 371 in human CBG [14] and all of them are conserved phylogenetically [15–17]. This therefore provides no clue to help identify the tryptophan within the steroid-binding site, but a comparison of the primary structures of human CBG and other members of the serine-proteinase inhibitor (serpin) superfamily [18] shows that Trp^{185} is invariably conserved, and that either a tryptophan or phenylalanine is present in a position analogous to Trp^{266} in human CBG. Consequently, these two residues probably represent essential components of the general conformation of serpins, rather than being located in the CBG steroid-binding site. The very close sequence similarity between human CBG and α_1-antitrypsin (AAT) [14] suggests that their tertiary structures are also similar, and substitution of residues in CBG with those in an analogous position in AAT should result in minimal perturbation of its overall conformation. With this in mind, we prepared a panel of human CBG cDNAs in which the codons for

*Correspondence to G. V. Avvakumov at London Regional Cancer Centre, 790 Commissioners Road East, London, Ontario, Canada, N6A 4L6.
Received 18 Oct. 1993; accepted 4 Feb. 1994.

the four tryptophan residues were individually substituted with those encoding phenylalanine, or the residue in the analogous position in AAT. These cDNAs were expressed in CHO cells, and the resulting mutants were examined with respect to their steroid-binding affinities and glycosylation.

EXPERIMENTAL

Construction and expression of CBG mutants

A cDNA for the human CBG precursor [14] was inserted into *Hind*III/*Xba*I-digested pSelect-1 (Promega, Madison, WI) and mutated, according to the protocol recommended by Promega, with oligonucleotide primers synthesized by the Molecular Biology Core Facility of the MRC Group in Fetal and Neonatal Health and Development (London, Ontario). These primers were designed to convert the codons for Trp (TGG) at positions 141, 185, 266, or 371 to a Phe (TTT) codon, and to convert the codons at position 141 and 371 to ACG (Thr) and AAG (Lys), respectively. The mutated cDNAs were sequenced to confirm the presence of only the targeted mutations [19], and subcloned into *Hind*III/*Xba*I-digested pRc/CMV (Invitrogen, San Diego, CA) for expression in CHO cells [12]. After selection in the presence of Geneticin (Gibco/BRL, Burlington, Ontario), stably-transformed cells were grown to near confluence and then cultured for 2–4 days in Dulbecco's modified Eagle medium containing 100 nM cortisol.

Binding assays and immunochemical analysis

The steroid-binding capacity of recombinant proteins in culture medium was determined by saturation analysis using [^{3}H]cortisol as labelled ligand [20], and their affinity for cortisol was measured by Scatchard analysis [21]. Their immunochemical properties were examined by radioimmunoassay (RIA) using a rabbit anti-human CBG antiserum, and ^{125}I-labelled [22] human CBG that was purified as described previously [23]. To determine the concentrations of immunoreactive CBG in samples of culture medium, appropriate dilutions of a human serum sample containing a known amount of CBG were used to construct the calibration curve. The characteristics of this RIA, including its accuracy, precision and sensitivity, have been extensively described elsewhere [23].

Western blotting

Polyacrylamide gel electrophoresis (4% stacking gel and 10% resolving gel) in the presence of SDS (SDS–PAGE) was performed according to Laemmli [24], and proteins were transferred to a Hybond-ECL (Amersham, Oakville, Ontario) membrane by electroblotting [25] 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).

Concanavalin A (Con A) chromotography

Culture medium (0.5 ml, 0.2–2 pmol immunoreactive CBG) was applied onto a 1.5 ml Con A–Sepharose (Pharmacia, Baie d'Urfé, Quebec) column, pre-equilibrated in 50 mM Tris, pH 7.5, 500 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ (Con A buffer), followed by 0.1 ml of this buffer. After a 5 min delay to allow glycoproteins to interact with the lectin, elution was performed with 4.5 ml Con A buffer, followed by 6 ml Con A buffer containing 100 mM methyl-α-D-mannopyranoside. Fractions (1.5 ml) were collected and analyzed by RIA.

RESULTS AND DISCUSSION

The side chain of tryptophan has a bulky, planar indole group that cannot be substituted by any other amino acid, and we chose initially to replace each of the tryptophan residues in human CBG with a phenylalanine largely because it also has a hydrophobic, aromatic side-chain. These mutations influenced the production of recombinant CBG in different ways (Table 1): a Phe at positions 141, 185 and 266 decreased the amount of immunoreactive CBG secreted into the culture medium, while the W371F mutant was secreted at a level similar to that of wild-type CBG. The codons for Trp^{141} and Trp^{371} were also mutated to introduce substitutions that correspond to amino acids in analogous positions in AAT. In the case of the W141T mutant, this resulted in a further decrease in secretion when compared to the W141F mutant, while the

Table 1. Effect of substituting tryptophan residues on the secretion and steroid-binding activity of human CBG expressed in CHO cells

	CBG concentrations in culture medium		Affinity for cortisol	
Mutants	nM	%[a]	$K_a \times 10^{-8}$, M^{-1}	%[a]
Wild-type CBG	1.2[b]	100	9.2[b]	100
W141F	0.9	75	0.9	10
W185F	0.35	29	1.8	20
W266F	0.55	46	1.2	13
W371F	1.2	100	ND[c]	—
W141T	0.35	29	ND	—
W371K	1.1	92	ND	—

Data are expressed as means of 2 determinations unless otherwise indicated.

[a]Corresponding values for wild-type CBG were taken as 100%.

[b]Mean of 8 experiments; coefficients of variation were 16.2 and 8.2% for the measurements of the concentration and K_a value, respectively.

[c]ND not detectable.

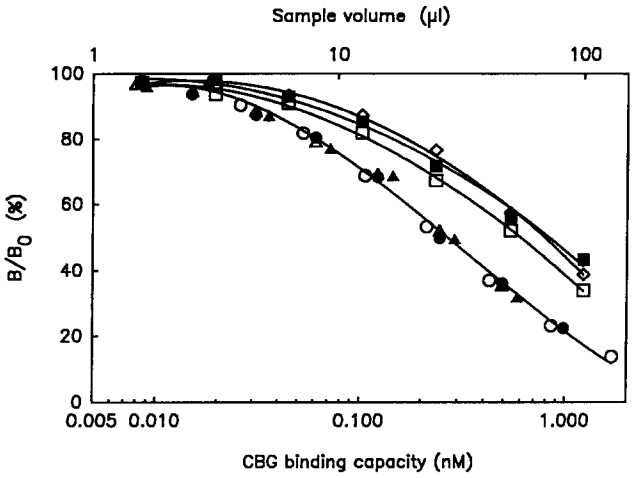

Fig. 1. Immunochemical properties of wild-type and mutant CBG. Inhibition of [^{125}I]CBG binding to anti-human CBG rabbit antiserum by wild-type human CBG (○) or mutants W141F (●), W141T (■), W185F (△), W266F (▲), W371F (□), W371K (◇) was expressed as percentage of B/B_0, where B and B_0 are the amounts of the tracer bound in the presence and absence of the competitor, respectively. The cortisol-binding capacity of wild-type CBG and CBG mutants with steroid-binding activity (see Table 1) was determined in samples of culture medium by Scatchard analysis. These values were used to construct dose–response curves (*lower scale*) which were essentially identical and are therefore represented by a single regression line. In the case of mutants W141T, W371F and W371K that lack steroid-binding activity, the individual inhibition curves were constructed on the basis of sample volume analyzed (*upper scale*).

W371K mutant was secreted in amounts similar to the W371F mutant and wild-type CBG (Table 1). The immunochemical properties of all the mutants were similar to those of wild-type CBG (Fig. 1), and this suggests that none of the tryptophan residues is involved in the formation of antigenic determinants. It also validates the use of the RIA for determining the concentrations of CBG mutants in culture medium.

The Western blot in Fig. 2 revealed that only mutant W266F differed in its apparent molecular mass, when compared to wild-type CBG. Its reduced electrophoretic mobility is probably due to a difference in glycosylation, and substitution with a phenylalanine at this position may result in a loosened conformation of the CBG molecule that allows more complete utilization of *N*-glycosylation sites and/or deeper processing of oligosaccharide chains. The latter possibility is supported by the fact that 69% of mutant W266F is not retarded during Con A chromatography, as compared to only 20% for wild-type CBG and 31% for mutant W371F, and this indicates a general shift from biantennary to more branched oligosaccharides in mutant W266F. This supports our observation that Trp^{266} may interact with the carbohydrate structure at Asn^{238} to limit oligosaccharide processing at this site [13].

Reduced secretion of proteins has been attributed to abnormalities in their folding during biosynthesis [26]. Substitution of tryptophan residues at positions 141, 185 and 266 in human CBG decreased its secretion from CHO cells, while mutants W371F and W371K were secreted at essentially the same levels as wild-type CBG (Table 1). This leads us to conclude that the 5-to-10-fold reduction in the steroid-binding affinity of mutants W141F, W185F and W266F (Table 1) may be due to minor deviations in their folding during biosynthesis. On the other hand, this cannot account for the lack of cortisol-binding to both mutants in which Trp^{371} was replaced by very different amino acids (Table 1). Trp^{371} is therefore most likely the residue that interacts with ring A of the steroid molecule [11], and that is consistent with the loss of high-affinity steroid binding to CBG after digestion with neutrophil elastase, which cleaves human CBG between residues 344 and 345 [27, 28]. Substitution of Trp^{141} with a hydrophilic residue (threonine) also resulted in a mutant with undetectable steroid-binding activity, suggesting that this tryptophan may also interact with the steroid molecule or participate in hydrophobic interactions with other residues that are important for the creation of the steroid-binding site.

In conclusion, Trp^{371} is probably located in the CBG steroid-binding site, and high-affinity steroid–protein interactions may also require the presence of Trp^{141}. This is in line with the fact that these two tryptophan residues are phylogenetically conserved, and are not present in other members of the serpin superfamily. The observations that the substitution of Trp^{266} results in altered glycosylation supports our previous suggestion that this residue is involved in intra-molecular carbohydrate–polypeptide interactions [13]. These findings should be taken into consideration when designing experiments to determine the structure of this glycoprotein and its steroid-binding domain.

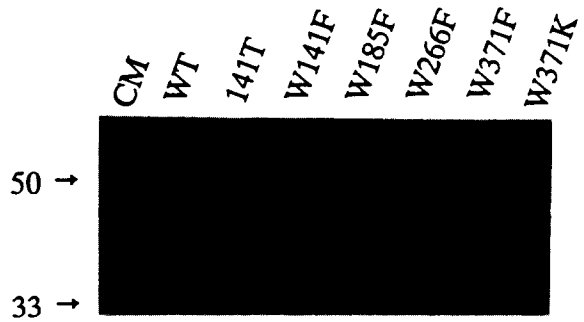

Fig. 2. A representative Western blot of wild-type CBG (WT) and CBG mutants (*as indicated above the lanes*) expressed in CHO cells. Proteins were resolved by 10% SDS–PAGE, transferred to a Hybond ECL membrane, and detected immunochemically. Conditioned medium from untransfected CHO cells (CM) represents a negative control, and the relative positions of pre-stained molecular weight (kDa) marker proteins are shown by arrows.

Acknowledgements—This work was supported by a grant from the Medical Research Council of Canada. G.L.H. is an Ontario Cancer Treatment and Research Foundation Scholar. The technical assistance of Susan Warmels-Rodenhiser and Allen Grolla is gratefully acknowledged. We also thank Gail Howard and Denise Hynes for their excellent secretarial help.

REFERENCES

1. Hammond G. L.: Molecular properties of corticosteroid binding globulin and the sex-steroid binding proteins. *Endocrine Rev.* **11** (1990) 65–79.
2. Rosner W.: The functions of corticosteroid-binding globulin and sex hormone-binding globulin: recent advances. *Endocrine Rev.* **11** (1990) 80–91.
3. Strel'chyonok O. A. and Avvakumov G. V.: Specific steroid-binding glycoproteins of human blood plasma: novel data on their structure and function. *J. Steroid Biochem.* **35** (1990) 519–534.
4. Le Gaillard F., Racadot A., Aubert J. P. and Dautrevaux M.: Modification of human transcortin by tetranitromethane. Evidence for the implication of a tyrosine residue in cortisol binding. *Biochimie* **64** (1982) 153–158.
5. Le Gaillard F. and Dautrevaux M.: Affinity labelling of human transcortin. *Biochim. Biophys. Acta* **495** (1977) 312–323.
6. Khan M. S. and Rosner W.: Investigation of the binding site of human corticosteroid-binding globulin by affinity labeling *J. Biol. Chem.* **252** (1977) 1895–1900.
7. Defaye G., Basset M., Monnier N. and Chambaz E. M.: Electron spin resonance study of human transcortin thiol groups and binding site topography. *Biochem. Biophys. Acta* **623** (1980) 280–294.
8. Stroupe S. D., Harding G. B., Forsthoefel M. W. and Westphal U.: Kinetic and equilibrium studies on steroid interaction with human corticosteroid-binding globulin. *Biochemistry* **17** (1978) 117–182.
9. Akhrem A. A., Avvakumov G. V., Kukushkina I. I. and Prishchepov A. S.: Tryptophan in the steroid-binding site of transcortin. *Bioorg. Khim.* **4** (1978) 421–423.
10. Akhrem A. A., Sviridov O. V., Strel'chyonok O. A. and Prishchepov A. S: Study of steroid-binding site in human transcortin using tryptophan-specific reagents. *Bioorg. Khim.* **7** (1981) 662.
11. Akhrem A. A., Sviridov O. V. and Strel'chyonok O. A.: Specific interactions in transcortin-steroid complex. *Bioorg. Khim.* **5** (1979) 711–720.
12. Avvakumov G. V., Warmels-Rodenhiser S. and Hammond G. L.: Glycosylation of human corticosteroid binding globulin at Asn-238 is necessary for steroid binding. *J. Biol. Chem.* **268** (1993) 862–866.
13. Avvakumov G. V. and Hammond G. L.: Glycosylation of human corticosteroid-binding globulin. Differential processing and significance of carbohydrate chains at individual sites. *Biochemistry* **33** (1994) in press.
14. 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.: Primary structure of human corticosteroid binding globulin, deduced from hepatic and pulmonary cDNAs, exhibits homology with serine protease inhibitors. *Proc. Natn. Acad. Sci. U.S.A.* **84** (1987) 5153–5157.
15. Hammond G. L., Smith C. L. and Underhill D. A.: Molecular studies of corticosteroid binding globulin structure, biosynthesis and function. *J. Steroid Biochem. Molec. Biol.* **40** (1991) 755–762.
16. Scrocchi L. A., Hearn S. A., Han V. K. M. and Hammond G. L.: Corticosteroid-binding globulin biosynthesis in the mouse liver and kidney during postnatal development. *Endocrinology* **132** (1993) 910–916.
17. Berdusco E. T. M., Jacobs R. A., Hammond G. L., Akagi K. and Challis J. R. G.: Glucocorticoid-induced increase in plasma corticosteroid-binding globulin levels in fetal sheep is associated with increased biosynthesis and alterations in glycosylation. *Endocrinology* **132** (1993) 2001–2008.
18. Huber R. and Carrell R. W.: Implications of the three-dimensional structure of α_1-antitrypsin for structure and function of serpins. *Biochemistry* **28** (1989) 8951–8966.
19. Sanger F., Nicklen S. and Coulson A. R.: DNA sequencing with chain-terminating inhibitors. *Proc. Natn. Acad. Sci. U.S.A.* **74** (1977) 5463–5467.
20. Hammond G. L. and Lähteenmäki P. L. A.: A versatile method for the determination of serum cortisol binding globulin and sex hormone binding globulin binding capacities. *Clin. Chim. Acta* **132** (1983) 101–110.
21. Scatchard G.: The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51** (1949) 660–672.
22. Fraker P. J. and Speck J. C.: Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* **80** (1978) 849–857.
23. Robinson P. A., Langley M. S. and Hammond G. L.: A solid-phase radioimmunoassay for human corticosteroid binding globulin. *J. Endocr.* **104** (1985) 259–267.
24. Laemmli U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** (1970) 680–685.
25. Towbin H., Staehelin T. and Gordon J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natn. Acad. Sci. U.S.A.* **76** (1979) 4350–4354.
26. Marquardt T. and Helenius A.: Misfolding and aggregation of newly synthesized proteins in the endoplasmic reticulum. *J. Cell Biol.* **117** (1992) 505–513.
27. Pemberton P. A., Stein P. E., Pepys M. B., Potter J. M. and Carrell R. W.: Hormone binding globulins undergo serpin conformational change in inflammation. *Nature* **336** (1988) 257–258.
28. Hammond G. L., Smith C. L., Paterson N. A. M. and Sibbald W. J.: A role for corticosteroid-binding globulin in delivery of cortisol to activated neutrophils. *J. Clin. Endocr. Metab.* **71** (1990) 34–39.