Human corticosteroid binding-globulin (CBG) is a plasma glycoprotein that binds and regulates the biological activity of glucocorticoids and progesterone. Carbohydrates comprise ~25% of its molecular mass being represented by bi- and triantennary N-linked oligosaccharides of the N-acetyllactosamine type. To assess the impact of these carbohydrate chains on CBG production and steroid binding, we mutated a human CBG cDNA so that the six consensus sites for N-glycosylation in the CBG polypeptide were eliminated individually and in various combinations. Expression of the mutant cDNAs in Chinese hamster ovary cells showed that all consensus sites may be utilized during the CBG biosynthesis and that the immunoochemical properties of the recombinant glycoproteins are similar to those of CBG isolated from human serum. Removal of sugar chains generally led to a reduction in the secretion of recombinant CBG, but complete removal of N-glycosylation sites did not prevent production or secretion of the protein. Our data indicate that an oligosaccharide linked to Asn238 is essential for steroid binding, and we suggest that an interaction between this sugar chain and the polypeptide may be essential for the creation of a high affinity steroid-binding site. In addition, concanavalin A chromatography of mutants containing only one N-glycosylation site at either Asn74 or Asn238 indicated that processing of the oligosaccharides at these positions is site-specific.

Experimental Procedures

Construction and Expression of Mutant CBG cDNAs—A cDNA for the human CBG precursor (7) was inserted into a HindIII/XbaI-digested pSelect-1 phagemid (Promega Corp.) and mutated to convert the codons for Asn (AAC or AAT) to Ala (GCA or GCT). Oligonucleotides (21 or 23 oligomers) complimentary 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 and were used as mutagenic primers. The CBG glycosylation sites were mutated individually, and cDNAs containing four or more mutations were constructed by combining appropriate portions of the individually mutated cDNAs. The mutated cDNAs were sequenced (15) to confirm by both biantennary and triantennary N-linked oligosaccharides of the N-acetyllactosamine type (4), and their relative proportions vary with differences in physiological state (5). There are six consensus sites for N-glycosylation (6) in the cDNA-deduced amino acid sequence of human CBG (7), and this suggests that one of these sites is not functional or that some of them are only partially utilized.

Carbohydrates associated with CBG may have diverse biological functions. For instance, terminal sialic acid residues are important for CBG survival in the circulation (8), and cortranslational glycosylation seems to be essential for the proper folding of newly synthesized CBG (9). Specific interactions between CBG and the plasma membranes of steroid-responsive tissues have also been reported (2, 10), and this suggests that CBG may target steroid hormones to specific cells. Furthermore, carbohydrates influence its interaction with plasma membranes of the human liver (11) and the syncytiotrophoblast (12), with N-acetylneuraminyl and α-mannosyl residues being of primary importance in this process (13). It has been reported that enzymatic deglycosylation of CBG does not influence its steroid binding (14), but the exoglycosidases used for this purpose do not completely remove carbohydrates attached to asparagine residues. In addition, these types of studies do not take into account that individual sugar chains are likely to affect glycoprotein structure and function in very different ways. Therefore, to study the biological significance of individual oligosaccharides linked to CBG, we modified a human CBG cDNA by site-directed mutagenesis so that consensus sites for N-glycosylation were systematically eliminated individually and in various combinations. These mutant cDNAs were expressed in Chinese hamster ovary (CHO) cells, and the biochemical and immunochemical properties of the recombinant proteins were studied.

Human corticosteroid-binding globulin (CBG) is a plasma glycoprotein that binds cortisol and progesterone with high affinity and acts to regulate their biological activities in extracellular compartments (1, 2). Carbohydrates comprise ~25% of its molecular mass, and there are about 5 moles of oligosaccharides per mole of CBG isolated from both normal donor and human pregnancy serum (3). More detailed analyses have indicated that these carbohydrates are represented by both biantennary and triantennary N-linked oligosaccharides of the N-acetyllactosamine type (4), and their relative proportions vary with differences in physiological state (5).

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Printed in U.S.A.

Glycosylation of Human Corticosteroid-binding Globulin at Asparagine 238 Is Necessary for Steroid Binding

(Received for publication, August 11, 1992)

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CHO, Chinese hamster ovary; RIA, radioimmunoassay; PAGE, polyacrylamide gel electrophoresis.

This work was supported by grants from the Medical Research Council of Canada and the Arcangelo Rea Family Foundation. The abbreviations used are: CBG, corticosteroid-binding globulin; CHO, Chinese hamster ovary; RIA, radioimmunoassay; PAGE, polyacrylamide gel electrophoresis.

* This work was supported by grants from the Medical Research Council of Canada and the Arcangelo Rea Family Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Carbohydrates associated with CBG may have diverse biological functions. For instance, terminal sialic acid residues are important for CBG survival in the circulation (8), and cortranslational glycosylation seems to be essential for the proper folding of newly synthesized CBG (9). Specific interactions between CBG and the plasma membranes of steroid-responsive tissues have also been reported (2, 10), and this suggests that CBG may target steroid hormones to specific cells. Furthermore, carbohydrates influence its interaction with plasma membranes of the human liver (11) and the syncytiotrophoblast (12), with N-acetylneuraminyl and α-mannosyl residues being of primary importance in this process (13). It has been reported that enzymatic deglycosylation of CBG does not influence its steroid binding (14), but the exoglycosidases used for this purpose do not completely remove carbohydrates attached to asparagine residues. In addition, these types of studies do not take into account that individual sugar chains are likely to affect glycoprotein structure and function in very different ways. Therefore, to study the biological significance of individual oligosaccharides linked to CBG, we modified a human CBG cDNA by site-directed mutagenesis so that consensus sites for N-glycosylation were systematically eliminated individually and in various combinations. These mutant cDNAs were expressed in Chinese hamster ovary (CHO) cells, and the biochemical and immunochemical properties of the recombinant proteins were studied.

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Construction and Expression of Mutant CBG cDNAs—A cDNA for the human CBG precursor (7) was inserted into a HindIII/XbaI-digested pSelect-1 phagemid (Promega Corp.) and mutated to convert the codons for Asn (AAC or AAT) to Ala (GCA or GCT). Oligonucleotides (21 or 23 oligomers) complimentary 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 and were used as mutagenic primers. The CBG glycosylation sites were mutated individually, and cDNAs containing four or more mutations were constructed by combining appropriate portions of the individually mutated cDNAs. The mutated cDNAs were sequenced (15) to confirm...
that only targeted mutations had occurred, and they were subcloned into a HindIII/XbaI-digested pRc/CMV eucaryotic expression vector (Invitrogen) for expression in CHO (CHO pro+) wild-type) cells (16). After selection in the presence of the neomycin analogue, Geneticin (GIBCO/BRL), stably transformed cells were washed twice with PBS to remove fetal calf serum and were then cultured in Dulbecco's modified Eagle's medium containing 100 nm cortisol for 2 days.

**CBG Assays**—The concentrations of CBG in culture media were determined using a radioimmunoassay (RIA) with a rabbit anti-human CBG antiserum, 125I-labeled CBG (specific activity 5–10 μCi/μg) prepared by iodination (17) of purified human CBG (18) using carrier-free Na125I (Amersham International), and appropriate dilutions of a human serum sample with known CBG concentration for the calibration curve (18). Separation of bound and free 125I-CBG was achieved by incubation with kaolin-conjugated secondary antibody (kindly provided by Farmos Diagnostica), followed by centrifugation and aspiration of the supernatant. The steroid-binding capacity of the recombinant CBG in the culture media was measured by saturation analysis using [1H]cortisol (55.8 Ci/mmol; Du Pont) as a labeled ligand and dextran-coated charcoal as a separation agent (19), and their affinity for cortisol was measured by Scatchard analysis (19, 20) after appropriate concentration (up to 5-fold) of culture media using Centricon microconcentrators (Amicon). The affinity constants \( K_d \) reported are the mean values of two independent experiments.

**Western Blots**—Polyacrylamide gel (4% stacking gel and 10% resolving gel) electrophoresis in the presence of SDS (SDS-PAGE) was performed according to Laemmli (21), and proteins were transferred to Hybond-ECL (Amersham International) membranes by electro blotting at 150–200 mA for 2 h. The blots were blocked and incubated with the anti-human CBG antiserum (1:500 diluted), and immunoreactive proteins were visualized with the ECL Western blotting system (Amersham Corp.).

**Concanavalin A Chromatography**—Culture medium (1 ml) was applied onto 2-ml concanavalin A-Sepharose (Pharmacia) columns, preequilibrated in the starting buffer (50 mM Tris, pH 7.0, 0.5 M NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂), followed by 0.5 ml of the starting buffer. After a 15-min delay to allow glycoproteins to interact with the immobilized lectin, elution was performed with 6 ml of starting buffer followed by 8 ml of the same buffer containing 0.15 M methyl-a-D-mannopyranoside. Fractions (1 ml) were collected and analyzed using the RIA described above.

**RESULTS**

**Effect of Glycosylation on Production of Recombinant CBG**—Table I illustrates the location of consensus sites for N-glycosylation in wild-type (Table I, 1) and mutant (Table I, 2–15) forms of human CBG and shows the levels of immunoreactive CBG in the culture medium from cells grown to confluence. Repeated transfection of CHO cells with plasmids containing the wild-type and mutated CBG cDNAs gave virtually the same results (not shown). The production of CBG mutants was lower than that of the wild-type glycoprotein, with the exception of the mutant which lacked consensus site V (Table I, 8), and was markedly decreased when all six glycosylation sites were eliminated (Table I, 15).

**Immunoreactivity and Steroid-binding Activity of Recombinant CBG**—Results presented in Fig. 1 indicate that the immunochemical properties of mutant proteins were very similar, and their dose-response curves parallel those of wild-type recombinant CBG and CBG in diluted human serum.

When the concentrations of immunoreactive CBG in culture media were compared to their corresponding cortisol-binding capacity measurements (Fig. 2), it was apparent that removal of glycosylation sites I, III, V, and VI had little or no effect on the binding activity of CBG. Substitution of Asn\(^{14} \) with Gin (glycosylation site II; Table I, 3) resulted in a substantial decrease in the ability of CBG to bind cortisol, but alternative mutation of this glycosylation site, by substitution of Thr\(^{16} \) with Ala, resulted in a CBG mutant (Table I, 4) with a binding capacity that corresponded closely to its concentration measured by RIA (Fig. 2). Scatchard analysis also revealed that its equilibrium association constant \( K_a = 1.5 \times 10^5 \text{ M}^{-1} \) for cortisol was similar to that of wild-type CBG (Fig. 3).

By contrast, mutation of glycosylation site IV by substitution of either Asn\(^{206} \) with Gln or Thr\(^{206} \) with Ala (Table I, 6, 7, 10, and 11) resulted in a complete loss of steroid-binding activity (Fig. 2). In addition, the CBG mutant that contains only one consensus site at this position (Table I, 14) was clearly able to bind cortisol \( (K_a = 1.3 \times 10^5 \text{ M}^{-1}) \), and when glycosylation site II was also present (Table I, 12), an increase in steroid-binding affinity \( (K_a = 1.9 \times 10^5 \text{ M}^{-1}) \) was observed (Figs. 2 and 3). However, the mutant containing only glycosylation site II (Table I, 13) had no steroid-binding activity despite a high level of immunoreactive CBG in the culture medium (Fig. 2). No steroid-binding activity was found in culture medium containing a CBG mutant with no consensus sites for N-glycosylation (Table I, 15), even after concentration of the medium by ultrafiltration (Fig. 2, point 16).

**SDS-PAGE of Recombinant Proteins**—Aliquots of culture media were analyzed by SDS-PAGE and immunoreactive CBG was detected by Western blotting (Fig. 4). The electrophoretic mobility of the mutant proteins increased in proportion to the number of glycosylation sites eliminated, and

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Human CBG Glycosylation

**FIG. 1. Immunochemical properties of wild-type and mutant recombinant CBG compared to CBG purified from human serum.** Inhibition of the binding of 125I-CBG to anti-human CBG rabbit antisera by serially diluted human serum or culture medium was assessed and expressed as a percentage of B/Bo, where B and Bo are the amounts of 125I-CBG bound in the presence and absence of competitor, respectively. Antisera was diluted either 1:5,000 (A and B) or 1:25,000 (C). Determinations were carried out in duplicate. Recombinant proteins are numbered as in Table I, A, serum CBG (+, upper scale); O, 1; ●, 2; △, 3; ▲, 4; □, 5; ■, 6; ▼, 7; □, 8; ○, 9. B, O, 1; ●, 10; Δ, 11; △, 12; □, 13; ■, 14; C, ○, 1; ●, 15.

**FIG. 2. Comparison between the concentration of immunoreactive CBG and cortisol-binding capacity of culture media.** The concentrations and cortisol-binding capacity of CBG in culture media were determined using RIA and saturation analysis, respectively. Data points correspond to the recombinant proteins as numbered in Table I; except point 16 which corresponds to completely deglycosylated CBG measured after a 6-fold concentration.

**FIG. 3. Scatchard analysis of cortisol binding to wild-type and mutant CBG.** Culture media containing either wild-type (●) or mutants (as numbered in Table I) 4 (□), 14 (■), or 12 (▼) were concentrated by ultrafiltration (2-5-fold), and aliquots (100 μl) were incubated with increasing amounts of [3H]cortisol (2,500-150,000 cpm/tube) in the presence (nonspecific binding) or absence (total binding) of 1,000-fold excess of unlabeled cortisol for 1 h at ambient temperature followed by 30-min incubation at 0 °C. The bound and free tracer was separated using dextran-coated charcoal, and bound [3H]cortisol was measured (19).

**DISCUSSION**

N-Linked glycosylation is a common post-translational modification of proteins in eucaryotic cells, and expression of CBG mutants lacking specific carbohydrate chains in CHO cells has enabled us to begin to define their biological significance. In general, the production of mutant proteins was lower than that of the wild-type recombinant CBG, was markedly decreased by elimination of all N-glycosylation sites, and is consistent with the concept that carbohydrates represent a "passport for secretion" (23). However, there was no correlation between the number of N-glycosylation sites and the level of glycoprotein production as reported for recombinant erythropoietin (24). Thus, a single carbohydrate chain attached to either Asn14 (site II) or Asn158 (site IV) was reflected a decrease in the molecular mass of CBG consistent with the removal of N-linked oligosaccharides of the N-acetyllactosamine type. Elimination of the consensus sites for N-glycosylation also resulted in a reduction in the size heterogeneity of recombinant proteins (Fig. 4), and this was particularly evident in the case of mutants containing only one site (lanes 13 and 14) or none at all (lane 15). It is noteworthy that CBG mutants with the same number of glycosylation sites migrate with slightly different mobilities (Fig. 4; lanes 2-9, 13, and 14), and this suggested that the structures of carbohydrate chains attached to particular sites were different.

**Lectin Affinity Chromatography**—To ascertain whether the difference in electrophoretic mobilities of mutants with similar numbers of glycosylation sites may reflect the attachment of carbohydrate chains with different structures, we studied the interaction of mutants containing only glycosylation site II or IV (Table I, 13 and 14, respectively), with concanavalin A. This lectin recognizes differences in the branching of oligosaccharides of the N-acetyllactosamine type (22), and concanavalin A affinity chromatography revealed that the mutant containing only consensus site II lacks glycoforms capable of interacting with concanavalin A, while the mutant containing only glycosylation site IV comprises almost exclusively lectin-retarded glycoforms (Fig. 5).
Human CBG Glycosylation

![Graph](image)

**Fig. 4.** SDS-PAGE of recombinant CBG. Recombinant proteins in the culture medium (0.2-0.8 ng/well) were separated electrophoretically in a 10% polyacrylamide gel in the presence of SDS, electroblotted onto Hybond-ECL nitrocellulose membrane and visualized as described under “Experimental Procedures.” Arrows indicate the positions of prestained protein standards: 106,000, phosphorylase B; 80,000, bovine serum albumin; 49,500, ovalbumin; 32,500, soybean trypsin inhibitor. The culture medium containing completely deglycosylated CBG was concentrated by ultrafiltration approximately 6-fold prior electrophoresis. Lanes correspond to the recombinant proteins as numbered in Table I; lane c, culture medium from wild-type CHO cells.

**Fig. 5.** Concanavalin A chromatography of CBG mutants containing only one consensus site for N-glycosylation. Culture media from mutants 13 (open bars) and 14 (shaded bars), as numbered in Table I, were chromatographed on concanavalin A-Sepharose as described in the text. The concentration of CBG in chromatographic fractions (1 ml) was determined using RIA and expressed as a percentage of the total immunoreactive CBG eluted from the column. Arrow indicates the beginning of the elution with methyl-α-D-mannopyranoside.

sufficient for secretion but removal of glycosylation site IV, individually or in combination with glycosylation site II, consistently resulted in lower levels of CBG in the culture medium. These data suggest that carbohydrates are not essential for CBG production and/or secretion, but their attachment at particular locations obviously influences these processes.

The immunochemical properties of wild-type and mutant forms of CBG secreted by CHO cells were identical to those of CBG in serum. This indicates our measurements of CBG concentration by RIA are accurate, and that the epitopes recognized by the antiserum are not influenced by carbohydrates. By comparing the mass concentrations of various CBG mutants determined by RIA with their cortisol-binding capacities, measured using an assay that is adversely affected by abnormalities in steroid-binding affinity (25, 26), we were able to deduce that the carbohydrate chain attached to Asn238 (glycosylation site IV) is essential for steroid binding. This was confirmed by disrupting the consensus site at this position in two different ways, namely by substitution of Asn238 with Gln, and Thr240 with Ala. Furthermore, when this consensus site was the only one present, the mutant CBG bound cortisol albeit with a relatively low affinity. Substitution of Asn14 with Gln (glycosylation site II) also decreased steroid-binding activity, but this is not related to the absence of a carbohydrate chain because alternative disruption of this consensus site resulted in a mutant with an affinity for cortisol identical to wild-type recombinant CBG. Glycosylation sites II and IV are conserved in CBG from several species, and carbohydrates at these positions may therefore be structurally or functionally important (27). In sheep, consensus site IV is conserved while site II is lacking, and this further supports our data indicating that glycosylation at site IV is necessary for steroid binding.

It has been claimed that removal of carbohydrates from human CBG by exoglycosidases does not affect steroid binding (14), but this treatment did not result in complete deglycosylation (14). Unglycosylated human CBG produced by insect (SF9) cells in the presence of tunicamycin has been found to be completely inactive with the implication that cotranslational glycosylation is required for correct protein folding (9). However, tunicamycin nonspecifically alters the properties of cellular glycoproteins (28) and induces the synthesis of proteins that bind and prevent secretion of misfolded proteins (29). Furthermore, the SF9 cells produce glycoproteins containing only non-sialylated, high mannose sugar chains (30), which may not interact with the polypeptide in the same way as more highly processed oligosaccharides associated with CBG produced by mammalian cells.

The molecular sizes of CBGs containing single mutations were consistently lower than that of wild-type recombinant CBG, and thus all six N-glycosylation sites in the CBG polypeptide can be utilized. However, their pronounced size heterogeneity indicates that some sites are only partially utilized, and this is consistent with data indicating that glycosylation site VI is both partially and differentially utilized (31). Size heterogeneity may also be attributed to variations in the structure of N-linked carbohydrate chains attached to the same residues (32), and CBG is known to contain both biantennary and triantennary oligosaccharides (3). The biological significance of this is not known, but the liver is not the only site of CBG production (7), and different cells may glycosylate CBG in unique ways and thereby provide diversity with respect to its biological properties.

Microheterogeneity does not imply a random mixture of oligosaccharide structures attached to all N-glycosylation sites in the CBG molecule. By analogy, the proportions of bi-, tri-, and tetra-antennary chains linked to the N-glycosylation sites of human α1-acid glycoprotein differ significantly (33). This supports the “site-directed” model of oligosaccharide processing which postulates that the interaction between the glycan and polypeptide determines the processing pathway leading to a particular oligosaccharide structure (34). Our analyses of CBG further validate this model because removal of glycosylation sites reduced the size heterogeneity of recombinant proteins, and mutants containing similar numbers of glycosylation sites had different molecular masses. Furthermore, lectin affinity chromatography of CBG mutants containing only one sugar chain showed that concanavalin A-binding glycoforms preferentially associate with glycosylation site IV, while glycosylation at site II results in glycoforms that do not interact with this lectin. When considered together with the relative mobilities of these mutants during SDS-PAGE, these data are consistent with the presence of biantennary oligosaccharides at site IV and more branched oligosaccharides at site II.

The primary structure of human CBG is closely related to α1-antitrypsin (7), and the concept that interaction with the polypeptide is responsible for the restricted processing of the biantennary chain at site IV is supported by the fact that one of the three oligosaccharides associated with human α1-anti-
trypsin interacts with a Trp residue (35). Furthermore, it has been proposed that the most probable candidate for this interaction is the chain attached to Asn\(^{347}\) in \(\alpha_1\)-antitrypsin (35), which aligns with Asn\(^{238}\) (site IV) in human CBG (7). It should also be noted that a Trp residue is involved in the binding of steroid to human CBG (36), and an interaction between a Trp residue and the oligosaccharide chain attached to Asn\(^{238}\) may therefore be important for the creation of the CBG steroid-binding site.

Acknowledgments—We thank Gail Howard for secretarial assistance and Allen Grolla and David Dales for technical assistance.

REFERENCES