N-Glycosylation influences human corticosteroid-binding globulin measurements

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Abstract

Objective: Discrepancies in enzyme-linked immunosorbent assay (ELISA) measurements of human corticosteroid-binding globulin (CBG) using detection monoclonal antibodies that recognize an epitope (9G12) within its reactive center loop (RCL), versus an epitope (12G2) in a different location, have suggested that CBG with a proteolytically-cleaved RCL exists in blood samples. We have previously been unable to verify this biochemically, and sought to determine if N-glycosylation differences account for discrepancies in ELISA measurements of CBG.

Methods and Subjects: Molecular biological, biochemical and glycopeptide analyses were used to examine how N-glycosylation at specific sites, including at N347 within the RCL, affect CBG ELISA or steroid-binding capacity assay (BCA) measurements. Plasma from patients with congenital disorders of glycosylation (CDG) was also examined in these assays as examples of N-glycosylation defects.

Results: We demonstrate that an N-glycan at N347 within the CBG RCL limits the 9G12 antibody from recognising its epitope, whereas the 12G2 antibody reactivity is unaffected, thereby contributing to discrepancies in ELISA measurements using these two antibodies. Qualitative differences in N-glycosylation at N238 also negatively affect the steroid-binding of CBG in the absence of an N-glycan at N347 caused by a T349A substitution. Desialylation increased both ELISA measurements relative to BCA values. Similarly, plasma CBG levels in both ELISAs were much higher than BCA values in several CDG patients.

Conclusions: Plasma CBG measurements are influenced by variations in N-glycosylation. This is important given the increasing number of CDG defects identified recently, and because N-glycosylation abnormalities are common in patients with metabolic and liver diseases.
Introduction

Corticosteroid-binding globulin (CBG) transports steroids in the blood and regulates their access to tissues and cells (1,2). Crystal structure analyses show that CBG contains a single hydrophobic steroid binding site (3) that is predominantly occupied by cortisol in human plasma (4). Human CBG is also known as SERPINA6 and shares structural similarity with several SERPINA family members (5) that inhibit specific proteases by ensnaring them after proteolysis of an exposed reactive center loop (RCL), which is a structural characteristic of SERPINs (6). While CBG does not inhibit proteases, its RCL is cleaved by neutrophil elastase (7,8), chymotrypsin (9), and the bacterial protease, LasB (10). This causes a conformational change in CBG with the insertion of the amino-terminal portion of the cleaved RCL as a novel β-strand, and this disrupts its ability to bind steroids with high affinity (3). Neutrophil elastase cleavage of the RCL of human CBG is thought to increase the amounts of anti-inflammatory steroids that are not protein-bound at sites of inflammation, infection or tissue damage (1,2).

Human CBG contains six sites for N-glycosylation in the mature polypeptide sequence (5), which are partly occupied by an ensemble of complex carbohydrates (11) that contribute to its electrophoretic heterogeneity (12). Importantly, N-glycosylation at N347 within the RCL of human CBG modulates how efficiently proteases cleave the RCL (13), and evidence that the type of N-glycan at N238 in human CBG influences its steroid-binding properties suggests that this oligosaccharide interacts with amino acid residues as the nascent glycoprotein is modified in the endoplasmic reticulum and Golgi during synthesis (12). In addition to protecting CBG against proteolysis and influencing steroid binding, the N-glycans may limit its recognition by monoclonal antibodies through steric hindrance or altered epitope conformation. This is important given the frequency of N-glycosylation abnormalities associated with disease (14,15).

Enzyme-linked immunosorbent assays (ELISAs) have been developed for human CBG measurements in biological fluids utilizing monoclonal antibodies that recognize specific epitopes on the surface of the natively folded glycoprotein (16,17). Plasma CBG levels measured by one of these ELISAs, which uses a monoclonal antibody (12G2) against an epitope that is unperturbed by structural changes...
caused by RCL proteolysis (16), correlate well with measurements of CBG concentrations based on its steroid-binding capacity (18). However, plasma CBG levels determined using these two different assay methodologies do not always correspond with those obtained by an ELISA that uses a monoclonal antibody (9G12) raised against a synthetic RCL polypeptide. This has led to the proposition that low affinity forms of CBG exist in blood from some individuals as a result of RCL proteolysis (17). We recently questioned this assumption and demonstrated that low affinity forms of CBG with a cleaved RCL are undetectable in human blood samples under normal or pathological conditions (18). Building on the biochemical analyses in the latter report, we now present evidence that discrepancies in these ELISA measurements of plasma CBG reflect abnormalities in CBG N-glycosylation rather than a disruption in the epitope within the RCL upon its proteolysis by neutrophil elastase.

Subjects and Methods

Human Subjects

Plasma from healthy individuals in whom plasma CBG measurements were either concordant or discrepant in ELISA measurements (18) were used as controls for CBG assays or desialylation experiments. Anonymized plasma samples from patients (IRB 16-004682-06) with congenital disorders of glycosylation (CDG) were used for CBG measurements. Patient demographics, and their biochemical and genetic characteristics are in Table 1. Patients with CDG type I have a genetic defect involving the endoplasmic reticulum (PMM2-CDG), while patients with CDG type II have a disorder affecting the Golgi apparatus (TMEM165-CDG and ATP6V0A2-CDG). Patients with PGM1-CDG showed a combined defect (mixed type I and II type CDG). Patients B, D, E and H carried homozygous mutations. Additional details of patients are in the following reports: Patients A and F (19), Patient B (20), Patients C and G (21), Patients D and H (22), Patient E (23). Studies were approved by both the Tulane University IRB and Mayo Clinic IRB Committee. Consent has been obtained from each patient after full explanation of the purpose and nature of all procedures used.
Production of recombinant human CBG and its N-glycosylation mutants

To determine whether N-glycans influence human CBG measurements by ELISAs, we expressed wild-type human CBG and CBG mutants in which specific N-glycosylation sites had been disrupted (Fig. 1A) in Chinese hamster ovary (CHO) cell lines. In addition to the CBG N238 + N347, CBG T349A and CBG A256T mutants used in previous studies (12,24), two other N-glycosylation deficient human CBGs, CBG N347D and CBG N238 + T349A, were prepared using the QuikChange II kit (Agilent). The CBG N347D mutant was generated using a pRc/CMV expression vector containing the wild-type CBG cDNA (25) as the template, with a mutagenic oligonucleotide 5´ACCCTAgACCTGACGTCCAAGCCTTC (mutated nucleotide in lower case). In contrast, the CBG N238 + T349A mutant was produced from a pRc/CMV plasmid encoding CBG N238 (25) with 5´-GGGGTCACCCTAAACCTGgCGTCCAAGCCTATCATC TTGC (mutated nucleotide in lower case). Mutated CBG cDNA sequences were verified prior to transfection of CHO-S cells (Gibco #11619-012) or CHO mutant lines, Lec1 (ATCC CRL-1735) or Lec2 (ATCC CRL-1736), for recombinant protein expression (12). As previously observed (24), variability in the expression of CBG constructs in CHO cell lines is likely due to differences in the proportion of stably-transfected cells expressing the cDNA constructs rather than issues with synthesis of the mutants.

Measurements of CBG

A radio-ligand saturation method (26) was used to measure the steroid-binding capacity of CBG in plasma, culture medium, or in samples after steroid-affinity purification. This steroid-binding capacity assay (BCA) only detects and measures CBG molecules that bind steroids with high affinity in biological samples, and does not detect CBG after proteolytic cleavage of the RCL because this causes an ~10 fold reduction in steroid-binding affinity (7). Briefly, samples were diluted in phosphate buffered saline (PBS) and steroids were removed by incubation with dextran-coated charcoal (DCC) followed by centrifugation. Samples (100 µL) of the supernatants were then incubated with ~10 nM [3H]-corticosterone (PerkinElmer Life Sciences, Waltham, USA) for 1 h at room temperature (RT) and 30 min at 4°C in the absence or
presence of a 200-fold excess of corticosterone to monitor non-specific binding. After separation of free steroids by adsorption with DCC at 0°C for 10 min followed by centrifugation, CBG-bound [³H]-corticosterone in the supernatants was measured in a scintillation spectrophotometer. A modification of this protocol was used for Scatchard analysis to determine steroid-binding affinity constants (26).

The CBG levels in plasma and cell culture media were also determined by two ELISAs using the same immobilized rabbit anti-human CBG polyclonal antibody (27) and two different murine monoclonal antibodies for detection: 12G2, which recognizes a surface epitope of CBG that includes A256 (16,24) and 9G12, which was raised against a synthetic polypeptide (17) that spans the neutrophil elastase cleavage site between V344 and T345 in the RCL of CBG (Fig. 1B). Serum with a known CBG concentration determined by BCA, was used to prepare ELISA standard curves. For some experiments, plasma CBG was semi-purified by steroid-affinity chromatography (27) prior to BCA and ELISA measurements.

For western blotting, CBG in plasma or in CHO culture media were subjected to denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE with 10% resolving gels) and proteins were transferred to PVDF membranes, as described (18). Briefly, blots were blocked with 5% milk-PBST and incubated overnight at 4°C with an affinity purified rabbit anti-human CBG antibody (18). Immuno-reactive CBG was detected using horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Sigma-Aldrich, St Louis, MO) and ECL reagent using an ImageQuant LAS4000 (GE Health Care).

**Enzymatic removal and modification of N-glycans**

Purified CBG (>90% purity, Affiland, Liége, Belgium) was reconstituted and 10 µg in 20 µL Tris-HCL, pH 7.5 was digested at 37°C with 5 U of PNGase F (Sigma-Aldrich, P7367) in a 30 µL reaction volume to remove N-glycans. After 1.5 h, 15 µL was removed from the incubation and frozen, while the remainder frozen after 18 h digestion. Sialidase A (Prozyme, Hayward, CA, U.S.A.) was used to desialylate CBG in plasma samples to determine how terminal sialic acid residues influence ELISA measurements of CBG. Desialylation was performed using 10 µL of plasma (1:10 in PBS) incubated (2 h at 37°C) with 10 mU
sialidase A in 20 µL. The PNGase F treated and desialylated samples were analyzed by western blotting and ELISAs, as above.

**Immuno-affinity purification of CBG and preparation for glycopeptide analysis**

Immunoaffinity columns were prepared by covalently coupling either purified polyclonal anti-CBG, the RCL-unspecific (12G2) monoclonal antibody, or the RCL-specific (9G12) monoclonal antibody, to cyanogen bromide-activated Sepharose 4B (Sigma-Aldrich). For this purpose, polyclonal anti-CBG IgG was purified by DEAE-Sephadex A50 chromatography using 10 mM phosphate buffer, pH 6.5, while the monoclonal antibodies were purified by Protein A agarose chromatography (Affi-Gel, Bio-Rad Laboratories, Hercules, CA). The columns were equilibrated with wash buffer (20 mM HEPES in 100 mM NaCl, pH 7.4) using gravity flow. Aliquots (20 µg) of human CBG (Affiland) were loaded onto each column, incubated for 15 min at RT before the non-retained fractions were collected. Columns were washed with wash buffer and the flow-through was pooled with the initial non-retained fractions. 1% (w/v) SDS was applied to each column, and incubated for 15 min at RT before the retained fraction was collected. Non-retained and retained CBG fractions were separated on a 12% SDS-PAGE gel, stained using Coommassie blue (Sigma) and the gel band intensities were measured using ImageJ software (http://imagej.nih.gov/ij/). The CBG bands were excised and washed with 50% (v/v) MeCN, 100 mM NH₄HCO₃ and 100% MeCN before incubated overnight with trypsin (1:50 w/w, enzyme:substrate, Sigma-Aldrich) at 37°C. The resulting peptides were extracted with 25mM NH₄HCO₃, 100% MeCN and 5% (v/v) formic acid. To determine the RCL occupancy, peptides were de-N-glycosylated overnight using 20U N-glycosidase F (Flavobacterium meningosepticum, Roche) at 37°C. CBG peptides were desalted using C₁₈ ZipTips (Merck Millipore, Germany) and eluted in 70% (v/v) MeCN in 0.1% (v/v) trifluoroacetic acid, dried, and re-dissolved in water for LC-MS/MS analysis.

**Analysis of CBG peptides by mass spectrometry (MS)**
The CBG peptides were separated on a 15 cm × 75 µm Reprosil-Pur C18AQ (particle size 3 µm, pore size 120 Å; Dr. Maisch GmbH, PhaseSep Pty Ltd, Australia) column using a Dionex 3500RS HPLC over a 60 min 0-50% solvent B gradient at a flow rate of 300 nL/min at 60°C (Solvent A: 0.1% (v/v) formic acid, Solvent B: 80% (v/v) MeCN in 0.1% (v/v) formic acid). We performed MS on an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Scientific) in positive polarity mode. The instrument parameters were: source voltage of +2.3 kV and capillary temperature at 275°C. The MS1 scans were acquired with a resolution of 60,000 in the Orbitrap mass analyzer with a scan range of \( m/z \) 300–2,000. Data-dependent tandem mass spectrometry (MS/MS) was performed using resonance activation collision induced dissociation (CID) with 35 NCE, activation time of 10 ms, AGC target of 5.0e4 and a maximum injection time of 300 ms. The MS1 and MS2 spectra were annotated using Xcalibur v3.0.63 software (Thermo Scientific). The relative abundances of the RCL peptides were calculated using the relative area-under-the-curve of Gaussian-smoothed extracted ion chromatograms (11,28).

**Results**

*Glycosylation of N347 limits CBG recognition in the 9G12 ELISA*

To determine whether the glycosylation of N347 within the RCL of human CBG influences its measurements in ELISAs, we expressed a CBG mutant in which T349 was substituted by alanine in wild-type CHO-S cells (Fig. 1A). This threonine lies just outside the epitope recognized by the 9G12 antibody (17). Thus, while the T349A substitution disrupts the N-glycosylation sequon in the RCL, it does so without altering the sequence of the 9G12 epitope that contains N347 (Fig. 1B). This was important because CBG N347D is undetectable in a 9G12 ELISA (Fig. 2A). In the latter experiment, we also included a CBG mutant (A256T) that is not recognised in the 12G2 ELISA (24). The Ala256 is located on the surface of human CBG in a position far from the RCL and this explains why the CBG A256T mutant is recognized appropriately in the 9G12 ELISA (Fig. 2A). Moreover, a synthetic peptide in which the asparagine (N347) resides within the 9G12 epitope is substituted with aspartic acid failed to interact with
9G12 (Fig. 2B). Intriguingly, prolonged PNGase F treatment of native CBG, which removes most of the \(N\)-linked glycans as evidenced by its increased electrophoretic mobility, resulted in comparable signals with the 9G12 antibody (Fig. 2C). This suggests that the \(N\)-glycan at N347 was not removed as it would have caused deamidation of N347 and loss of 9G12 recognition, and explains why plasma samples with discrepant CBG values in a 9G12 ELISA were previously unaffected by PNGase F treatment (29).

We also studied CBG mutants that only contain an \(N\)-glycosylation site at N238 in the presence (CBG N238 + N347) or absence (CBG N238 +T349A) of an \(N\)-glycosylation site at N347 (Fig. 1A). The N238 was retained in all mutants because \(N\)-glycosylation of this residue is required for formation of a high affinity steroid-binding site (25). The wild-type CBG and \(N\)-glycosylation deficient CBG mutants were expressed in glycosylation competent CHO-S cells or in CHO Lec1 cells that do not synthesize complex or hybrid \(N\)-glycoproteins, and sialylation deficient Lec2 cells (Fig. 3A).

The wild-type and \(N\)-glycosylation deficient CBGs secreted into culture medium by CHO-S cells and by Lec1 or Lec2 CHO cells that lack specific N-glycosyltransferases (Fig. 3A) were first semi-quantified by western blotting to assess their integrity, prior to steroid-binding capacity measurements (Fig. 3B). Western blot analysis demonstrated that the wild-type CBG and CBG mutants were produced by all three CHO cell lines with electrophoretic mobilities that reflect the expected number of \(N\)-glycosylation sites. In the media from all three CHO cell lines, the CBG N238 + N347 mutant was more heterogeneous in terms of its electrophoretic isoforms when compared with the CBG N238 + T349A mutant, and this was most apparent when comparing these mutant proteins produced by Lec1 cells (Fig. 3B). In the latter case, a doublet was observed for the CBG N238 + N347 mutant: one component of which has an electrophoretic mobility similar to the single band observed for the CBG N238 + T349A mutant with only one \(N\)-glycosylation site. Although amino acid substitutions can alter the mobility of CBG during SDS-PAGE (24), this suggests that one of the \(N\)-glycosylation sites in CBG N238 + N347 is not utilized. Remarkably, CBG N238 + T349A expressed in CHO-S and Lec1 CHO cells bound steroid normally, but lacked steroid-binding when expressed in Lec2 CHO cells (Fig. 3B).
In contrast to our ELISA measurements of CBG in plasma samples (18), the amounts of wild-type CBG produced by all three CHO cell lines were always greater when measured by the 9G12 ELISA than by the 12G2 ELISA (Fig. 3C). This discrepancy was similarly observed in ELISAs of the CBG mutant with N-glycosylation sites only at N238 and N347 (CBG N238 + N347), but it was enhanced in the CBG T349A mutant lacking an N-glycosylation site within the RCL, as well as in the CBG N238 + T349A mutant carrying only the N-glycan required for high affinity steroid-binding without the RCL N-glycan (Fig. 3C). These discrepancies in CBG ELISA measurements were consistent in the culture media harvested from all three CHO cell lines (Fig. 3C).

*Sialylation of N-glycans associated with CBG influences ELISA values*

We then enzymatically removed sialic acid residues from the N-glycans attached to CBG in two subsets of human plasma samples in which the initial ELISA measurements were either concordant or highly discrepant (Fig. 4A). As expected, western blotting showed that desialylation of CBG increased the electrophoretic mobility of the major CBG glycoforms (Fig. 4B). Desialylation of the N-glycans decorating CBG also resulted in significant increases in immunoreactivity in both 12G2 and 9G12 ELISAs, as compared to BCA values which remained unchanged. When examining the changes in 9G12 ELISA values following desialylation, increases in immunoreactivity were observed. The largest such increase occurred in samples in which the original ELISA values were concordant (Fig. 4C), and is attributed to qualitative and quantitative differences in glycosylation at N347. In comparison, the relative changes in CBG levels measured by the 12G2 ELISA after desialylation were similar in all samples, irrespective of whether they were originally concordant or highly discrepant.

*N-glycan occupancy of CBG N347 limits the interaction to RCL-specific antibodies*

To build further evidence that the glycosylation of N347 influences ELISA measurements of CBG, purified human CBG was further fractionated using different anti-human CBG antibody columns with unique immunorecognition properties. As expected, the entire populations of CBG glycoforms were
captured by the affinity columns conjugated with the polyclonal anti-CBG antibodies or the 12G2 monoclonal antibody, as indicated by the absence of CBG in the non-retained fractions (Fig. 5A). In contrast, the affinity columns conjugated with the RCL-specific 9G12 anti-CBG antibody only retained a sub-population of the CBG glycoforms. The relative intensities of the top and bottom CBG gel bands representing different glycoform populations of CBG were then measured in the non-retained and retained fractions from the affinity purifications of CBG relative to a non-purified CBG reference (Fig. 5B). This confirmed that 9G12 preferentially recognizes CBG glycoforms displaying a high gel mobility.

The presence of the occupied and unoccupied RCL peptides in these fractions was evidenced by 1Da mass difference introduced by the conversion of asparagine (N) at position 347 to aspartic acid (D) upon PNGase F removal of a glycan from this residue (3109.71 Da ($m/z$1037.57$^{3+}$) vs 3108.72 Da ($m/z$1037.24$^{3+}$), left inserts, Figs. 5D-E.), as well as the same molecular mass change in their fragment ions (y26$^{2+}$ ions, right inserts, Figs. 5D-E) and their distinct retention times (56.09 min vs 55.34 min, left inserts, Figs. 5D-E). Thus, these data indicate that the glycosylation status of N347 impacts the recognition of CBG by RCL-specific anti-CBG antibodies.

**Abnormally glycosylated CBG in plasma from CDG patients results in ELISA discrepancies**

To assess the effect of aberrant N-glycosylation on the ELISA measurements, we analyzed plasma CBG from eight patients (A-H) with CDG (Table 1). For these analyses we used a reference sample (C1 in Fig. 4) in which the 12G2 ELISA and binding capacity assay (BCA) measurements were concordant, while 9G12 ELISA values were much lower than the 12G2 ELISA or BCA measurements (18). To accomplish this, western blotting was first used to assess the relative abundance and electrophoretic properties of CBG in the patient plasma samples (A-H) as compared to the control (C1). The CBG in most of the CDG patient samples displayed a degree of electrophoretic heterogeneity similar to that observed for CBG in healthy individuals (7), e.g., in the C1 sample (Fig. 6A). However, the plasma CBG in some patients (C, G and H in Fig. 6A) was characterized by multiple isoforms of lower apparent molecular weight than the major ~60 kDa glycoforms observed in C1, consistent with quantitative or qualitative abnormalities in N-
glycosylation. Two of these patients (C and G) had a Type 1 CDG caused by biallelic pathogenic variants in *PMM2*, while the other (H) had a mixed Type 1 and Type 2 CDG due to biallelic pathogenic *PGM1* variants. The large number of CBG electrophoretic isoforms in the plasma of patient G was particularly interesting, as the major ~60 kDa CBG bands in the control (C1) and other CDG patient samples were absent in this sample. By Scatchard analyses, the affinities of CBG for corticosterone in CDG patient plasma samples (expressed as Kd values in nM), were similar to that in C1 (Fig. 6A).

We then compared the ELISA measurements of plasma CBG in the CDG patients with those obtained using the BCA (Fig 6B). This demonstrated that the 12G2 ELISA values in six patients (A, B, C, F, G and H) were 0.6-fold to 1.5-fold higher than the corresponding BCA values, but in the two other CDG patients (D and E) the 12G2 ELISA and BCA values were closely aligned, as in the control (C1). As in C1, the 9G12 ELISA values were lower than the BCA values in plasma samples from some CDG patients (A, B, D, E, and F) suggesting a reduced 9G12 epitope recognition of CBG in these samples. In the two Type 1 CDG patients (C and G), and the patient with a mixed Type 1 and Type 2 CDG (H), the 12G2 ELISA measurements of CBG exceeded the BCA values and their 9G12 ELISA values were either similar to or much higher that the BCA values. This discrepancy between ELISA and BCA values was particularly evident in the sample from patient G in whom the electrophoretic heterogeneity of CBG was most abnormal (Fig. 6A), and in this sample the 9G12 ELISA value was >4.5-fold higher than the BCA value and almost 2-fold higher than the 12G2 ELISA value (Fig. 6B).

These experiments were repeated using CBG semi-purified from patient plasma samples prior to the ELISA and BCA measurements. The results confirm that the 12G2 ELISA measurements of CBG in the CDG patient plasma samples either aligned with or were higher than BCA values, and again this was most apparent in the sample from patient G (Fig. 6C). Moreover, the 9G12 ELISA values of CBG were generally higher when compared to the BCA values for the purified CBG from patient samples and again this was greatly accentuated in the case of patient G (Fig. 6C).

**Discussion**
We set out to determine why 9G12 ELISA measurements of CBG in healthy individuals (17,29) and in some patients with sepsis (30) or various other diseases (31–33) are lower than corresponding 12G2 ELISA or BCA measurements (18,34). The fact that the 9G12 epitope within the RCL of human CBG contains the N347 glycosylation site that is not always fully utilized (13), and is positioned only two residues C-terminal of the neutrophil elastase cleavage site (Fig. 1B), suggested that this might modify the way the 9G12 antibody recognizes the RCL. To test this, we used different approaches to prevent or alter the N-glycosylation at N347 in the RCL. As hypothesized, our experiments demonstrate that CBG without an N-glycan in this position is recognized more effectively by the 9G12 monoclonal, and that the ability of this antibody to recognize its epitope is influenced by the complexity of the attached N-glycan. Establishing this molecular relationship cautions against drawing conclusions about the structural or functional properties of a protein analyte based on immunoassays that rely on a monoclonal antibody as the detection reagent. This is not without precedent, as glycosylation alters the recognition of epitopes by monoclonal antibodies in ELISAs of other glycoproteins, including MUC2 mucin (35) and the vitamin D-binding protein (36,37).

Unexpectedly, we also observed that disruption of the N-glycosylation sequon (NLT) within the RCL by a threonine-to-alanine substitution results in a complete loss of steroid-binding activity when the mutant proteins were expressed in Lec2 CHO cells, and that this occurred even in a mutant (CBG N238 + T349A) that is only N-glycosylated at N238. We know that the N-glycan at N238 in human CBG is essential for the formation of a high affinity steroid-binding site (12,25), and that mutation of N347 as in the CBG N347D mutant has no effect on steroid binding although it prevents glycosylation at this site (25). It is therefore remarkable that mutation of T349, which has the same effect, has such a detrimental effect on steroid binding because it lies within the exposed RCL and is unlikely to interact with amino acid residues within the steroid-binding site. Since this effect was not observed when the CBG T349A mutant was expressed in CHO-S cells or Lec1 cells that produce less complex N-glycan structures (Fig. 2A), or when the N347 in the RCL is mutated to disrupt the N-glycosylation site (12,25) we are left with the
possibility that structural differences in the oligosaccharide additions to N238 affect the steroid-binding activity of CBG through interactions with Thr349 or some other structural component of the RCL. Crystal structure and modelling of oligosaccharide chains attached to the glycosylation sites of rat CBG, which correspond to N238 and N347 in human CBG, predict that these N-glycans are in close proximity to each other (12). This might suggest that glycan-glycan interactions influence the folding of the protein during its synthesis and its acquisition of a high affinity steroid-binding site, but this is unlikely because the latter only requires N-glycosylation at N238 (25). Nevertheless, these observations stress the importance of considering the structures and molecular interactions of the carbohydrates decorating CBG when exploring the structure/function relationship(s) of this glycoprotein.

Our analyses of plasma CBG levels in patients diagnosed with CDGs are informative in several ways that may be diagnostically important. This is because >150 different types of CDG have been identified (14) that involve mutations in genes that are key components of the N-glycosylation machinery including PMM2, PGM1, TMEM165 and ATP6V0A2. Although many of these mutations are rare, >980 PMM2-CDG cases have been reported, and the disease frequency is estimated to be 1:100,000 (38). Moreover, some of these patients have an R141H substitution in phosphomannomutase 2 (PMM2) that is relatively common, with a carrier frequency of 1/79 in Dutch/Flemish and 1/60 in Danish populations, respectively (39). It is not clear how the efficiency of N-glycosylation in the liver is affected in heterozygous carriers of these mutations, but this may in part explain the discrepancies in ELISA measurements of plasma CBG in other patient groups as well as in cohorts of healthy individuals, as mentioned above.

Our CDG patient group included very severely affected patients with pathogenic nonsense variants in PMM2, PGM1, or ATP6V0A2, as well as a patient who was homozygous for a deep intronic pathogenic variant in TMEM165 (Table 1). The two PMM2-CDG patients are compound heterozygous for severe pathogenic missense variants (Table 1), including one patient (Patient C) who was heterozygous for two common PMM2 variants (C241S and R141H). It is therefore interesting that the electrophoretic heterogeneity of plasma CBG in these two patients is much more exaggerated than in other patients, with
the exception of Patient H who is homozygous for a rare mutation that causes an S338R substitution in phosphoglucomutase 1(PGM1). There is no information about the biochemical consequences of this particular PGM1 mutation but our western blotting experiment indicates that it disrupts the glycosylation of plasma CBG profoundly. Moreover, the ELISA values of plasma CBG in Patient H were either similar to or higher than the BCA measurements. By contrast, the CBG glycoform profile in plasma from Patient D was only marginally different from that in the control sample, and the ELISA values showed the same type of discrepancy as in the reference sample. This is interesting because Patient D is homozygous for the more common R515Q substitution that occurs within the substrate-binding loop of PGM1, which is known to influence the catalytic activity of this enzyme (40). Although the biochemical consequences of this particular mutation are not well understood, our data suggest that the PGM1 R515Q variant is less detrimental than the PGM1 S338R variant at least in terms of the N-glycosylation of proteins like CBG that are produced by the liver.

Plasma CBG levels determined by ELISAs in some CDG patients were higher than their corresponding BCA values, especially when the 12G2 monoclonal antibody was used. This was unexpected because BCA and 12G2 ELISA measurements of CBG displayed a high degree of correspondence in healthy individuals and acutely ill patients in our previous studies (18,34). We therefore conclude that the abnormal N-glycosylation of CBG in CDG patients either allows the protein to be more efficiently recognized by the monoclonal antibodies or that some CBG glycoforms bind steroid with abnormally low affinity, which is a possibility because the presence and composition of N-glycans at specific locations on the CBG molecule influence its steroid-binding properties (12,25). Interestingly, our data suggest that the N-glycan at N238 interacts with residues in the RCL region to influence the steroid-binding activity of CBG.

These observations highlight the limitations of methods used to measure CBG in biological samples. We have previously demonstrated that CBG variants with specific amino acid substitutions may either be undetectable in ELISAs using monoclonal antibodies (e.g., 12G2) because of a disruption in the epitope, or in the BCA because of a loss of steroid-binding activity (24,41–44). Our findings indicate that
abnormalities in $N$-glycosylation alter the recognition of CBG in ELISAs in different ways depending on the monoclonal antibody used as the detection reagent, but may also confound BCA measurements. These analytical limitations were evident in the limited cohort of CDG patients available for our studies, but alterations of $N$-glycosylation are common in numerous acquired diseases (15) and this needs to be considered when measuring plasma CBG measurements in patients with these conditions.
Declaration of Interest

The authors declare they have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions

GH and LAH designed the study. LAH, ZSB, MTA and JGL carried out experiments and analyzed the data, and GLH prepared the manuscript with input from LAH, ZSB, MTA, JGL, and EM. EM provided samples from CDG patients and participated in data analysis. All authors have approved the research and contents of the manuscript for publication.

Acknowledgements

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References


Figure Legends

Figure 1. Positions of N-glycans attached to human CBG and the CBG N-glycosylation mutants investigated, the CBG reactive center loop (RCL) sequence and 9G12 monoclonal antibody epitope. (A) Structural model demonstrating the extent of N-linked glycosylation of human CBG in its relaxed (R) conformation. In the R conformation, the CBG RCL has been cleaved and inserted into the core of the protein. The N-glycans shown are based on their reported most frequent compositions (11). The predicted N-glycan at Asn9 could not be added because this position is lacking in the crystal structure that the model is based on. The CBG protein is coloured light grey and glycans are coloured dark grey, with the exception of those attached to N238 and N347, which are coloured black. The steroid-binding site (dashed circle) is indicated, as are epitopes (black) recognized by monoclonal antibodies used in the ELISAs, i.e. A256 for the 12G2 ELISA and S341-L348 for the 9G12 ELISA, in the lower panel. In this representation, RCL-cleaved human CBG (PDB ID 4BB2) was utilized for in silico modeling of N-linked glycans, using the program GlyProt (http://glycosciences.de) and illustrations were created using the program PyMol (http://pymol.org). (B) Positions of the N-glycosylation sites in wild type (WT) human CBG and in the CBG N-glycosylation mutants studied, including CBG T349A that disrupts glycosylation at N347 within the RCL without directly affecting the 9G12 epitope. (C) The CBG RCL sequence with the N-glycosylation (N-L-T) sequon in bold. The 9G12 antibody epitope is underlined with an arrow.

Figure 2. N347 is a key recognition amino acid in the epitope for the RCL monoclonal antibody 9G12. (A) Wild type (WT) human CBG is recognised in both the 12G2 and 9G12 ELISAs, whereas the N347D mutant is undetectable using 9G12. The CBG A256T mutant served as a control as it is recognised by the 9G12 antibody and not the 12G2 antibody. Data are expressed as mean ± SD for n=3 technical replicates. (B) Chromogenic signals in horizontal duplicates in microtitre plate wells with RCL antibody 9G12 show immobilised RCL synthetic peptide is recognised and not displaced by either the control
(buffer only) or D347 synthetic peptide (STGVTLDLTSK) at 100µg/mL. This contrasts with complete displacement using N347 RCL peptide (STGVTLNLTSK) at 100µg/mL. (C) PNGase F treatment (+) of native CBG for 1.5h or 18h followed by SDS PAGE and western blotting with 9G12 or polyclonal antibodies show equivalent signals and mobility shifts compared to no treatment (-). This indicates some carbohydrate removal but not at N347 as deamidation to D347 would be an expected consequence with the absence of recognition by antibody 9G12. Positions of molecular weight markers (kDa) are on the left.

Figure 3. Loss of N347 glycosylation within the CBG RCL increases 9G12 ELISA values, irrespective of the types of N-glycosylation. (A) Examples of the type of N-glycosylation that can be expected to be attached to CBG expressed in fully glycosylation-competent (CHO-S) and partially deficient (Lec1 and Lec2) CHO cells (45). Lec1 cells lack a functional N-acetylglucosaminyltransferase-I, leading to homogenous high mannose N-glycosylation, whereas Lec2 cells lack the CMP-sialic acid Golgi transporter and are therefore unable to sialylate the expressed glycoproteins. (B) Differences in N-glycosylation on CBG influence its mobility during SDS-PAGE as assessed by western blotting. The CBG steroid-binding capacity (nM) as measured by BCA is shown for wild type (WT) CBG and the CBG glycosylation mutants applied to the western blot. Molecular weight markers in kDa are on the left of the blot (C) The WT CBG and glycosylation-deficient CBG mutants produced by CHO-S, Lec1 and Lec2 cells were measured using the 12G2 and 9G12 ELISA methods. The CBG concentrations in culture media were higher with the 9G12 ELISA when compared to the 12G2 ELISA. Abnormally high 9G12/12G2 ELISA ratios were observed for the CBG T349A mutant. Data are expressed as mean ± SD for n=3 technical replicates.

Figure 4. Desialylation increases the recognition of plasma CBG in the 12G2 and 9G12 ELISAs. (A) Comparison between the steroid-binding capacity assay (BCA) and 12G2 or 9G12 ELISA measurements of CBG in representative plasma samples in which the two ELISA measurements were either concordant
or discrepant. (B) Western blotting of the same plasma samples as in (A) shows that desialylation of CBG N-glycans increased the electrophoretic mobility of CBG. Plasma samples were either untreated (-) or treated (+) with sialidase A. Molecular weight markers in kDa are on the left of the blot. (C) Plasma CBG concentrations were measured by BCA, 12G2 and 9G12 ELISA and expressed as a percentage of the untreated (no sialidase) value for each sample. Data are expressed as mean ± SD for n = 3 technical replicates.

**Figure 5.** The N-glycan occupancy of CBG N347 negatively impacts the recognition of CBG by the RCL-specific antibody 9G12. (A) SDS-PAGE gel analysis of the affinity-purified human CBG using anti-human CBG antibody columns with different structural recognition features of CBG. All fractions were analysed on the same gel (only relevant lanes are shown) and the bands used for further analyses are indicated. (B) Relative intensities of the top and bottom CBG gel bands representing different glycoforms of CBG in: i) non-retained and ii) retained fractions from the affinity purification of CBG. Data are expressed as means ± SD for n=3 affinity purification replicates, *p < 0.05, **p < 0.01. (C) Degree of N-glycan occupancy of the RCL-located N347 site based on the relative abundance of the occupied and unoccupied peptides in the non-retained and retained CBG fractions using LC-MS/MS. The indicated RCL N347 occupancies are the mean calculated from two separate LC-MC/MS experiments. (D, E) Representative CID-MS/MS product ion spectra from the LC-MS/MS analyses of N-glycosidase F treated RCL peptides. A (formerly) occupied RCL peptide with a clear N to D (underlined) conversion (top spectrum) and an unoccupied RCL peptide presenting native N347 residue (bottom spectrum). The extensive y-ions series, including a zoom of the isotope pattern of the product ions confirming the indicated RCL peptide sequences (inserts on the right). The different elution times and precursor ion isotope patterns of the two variants of the RCL peptide have also been provided to build further support for the presence of the occupied and unoccupied RCL peptides (inserts on the left).
Figure 6. ELISA measurements of plasma CBG are affected by aberrant glycosylation in CDG patients. (A) Plasma CBG in CDG patients (A-H in Table 1) was analyzed by western blotting to assess their molecular properties and by Scatchard analysis using $[^3]$H-corticosteroid as labelled ligand to determine their steroid-binding affinity. Immuno-reactive CBG isoforms of lower molecular weight than in the C1 reference sample were observed in plasma from patients C, G and H, suggesting qualitative or quantitative alterations of the CBG N-glycans in these samples. In contrast, the steroid-binding affinity (Kd) of plasma CBG in the CDG patients were similar to that recorded for the C1 sample. (B) Comparison of 12G2 or 9G12 ELISA versus BCA measurements of CBG in plasma samples from CDG patients (A-H) and a reference sample (C1) in which the 9G12 ELISA measurement was discrepant. (C) The CBG in plasma samples used in (B) were steroid-affinity gel purified and re-analyzed by BCA, 12G2 and 9G12 ELISAs, and average (n=3-5 technical replicates) 12G2 or 9G12 ELISA measurements of CBG are compared as % of BCA values.
Table 1. Biochemical, demographic and genetic data for patients with biallelic pathogenic variants leading to CDG

<table>
<thead>
<tr>
<th>Patient</th>
<th>CDG type (biochemical classification)</th>
<th>Affected gene</th>
<th>Protein change due to pathogenic variants</th>
<th>Zygosity</th>
<th>Sex</th>
<th>Age (years)</th>
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<td>B</td>
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#c.792+182G>A deep intronic, homozygous mutation
A

![Bar graph showing ELISA results for WT, A256T, and N347D proteins. The x-axis represents the proteins, and the y-axis represents ELISA (nM). The graph shows 12G2 ELISA and 9G12 ELISA results.]

B

![Image of a gel showing bands for 9G12, STGVTL, DLTK, STGVTL, NLTSK, and control.]

C

![Western blot images showing bands for CBG and 9G12 proteins at different conditions (CBG - 1.5h, CBG + 1.5h, CBG - 18h, CBG + 18h). The x-axis represents kDa, and the y-axis represents the intensity of the bands.]

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**A**

![Diagram showing glycosylation patterns](image)

- **CHO-S**
- **Lec1**
- **Lec2**

**B**

**CBG-steroid binding capacity (nM)**

<table>
<thead>
<tr>
<th>kDa</th>
<th>WT</th>
<th>T349A</th>
<th>N238 + N347</th>
<th>N238 + T349A</th>
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**C**

**9G12/12G2 ELISA (%)**

- **CHO-S**
- **Lec1**
- **Lec2**
A) Non-retained vs Retained

LC-MS/MS occupancy analysis of 9G12 fractions (panel C-E)

B) i) Non-retained

Bottom band intensity
Top band intensity

nd
nd

ii) Retained

Polyclonal anti-CBG
12G2
9G12

Relative band intensity

CBG Reference
Polyclonal anti-CBG
12G2
9G12

C) (Formerly) Occupied RCL peptide

100%
80%
60%
40%
20%
0%

Rel abundance

Non-retained
Retained

D) Unoccupied RCL peptide

AVQLNEEGVTDAGSTGVRLTLSKPIILR

Relative band intensity

m/z

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