Supplementary data for:

N-GLYCANS MODULATE THE FUNCTION OF HUMAN CORTICOSTEROID-BINDING GLOBULIN

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Running head: Structure and Function of Human CBG N-glycans

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Legends for Supplementary figures

Supplementary figure 1. For site-specific identification and relative quantitation of CBG N-glycoforms, glycoproteomics LC-MS data was searched against this catalogue of observed and hypothetical CBG N-glycans using GlycospectrumScan. The catalogue was manually generated based on initial interpretation of LC-MS/MS glycomics and glycoproteomics data. In total 36 related glycoform compositions were generated covering sialylated/ non-sialylated and fucosylated/non-fucosylated complex type N-glycans.

Supplementary figure 2. Determination of the NeuAc linkage. XICs for \(m/z\) 1111.3 (A), \(m/z\) 820.3 (B), \(m/z\) 1439.3 (C) and \(m/z\) 1002.8 (D) have been generated for untreated (upper chromatograms) and \(\alpha_2,3\)-specific neuraminidase treated (lower chromatograms) CBG N-glycans (alditols). The abundant bi- and tri-antennary fully sialylated complex N-glycans eluting at retention time 55 min (A) and 65 min (C), respectively, are completely absent after enzyme digestion and are observed as the non-sialylated bi- and tri-antennary structures at retention time 45 min (B) and 52 min (D). Partially desialylated N-glycans are not present in the enzyme-treated sample as shown by lack of signal when making XIC for the corresponding \(m/z\) values (data not shown). It is likely that the doublet peaks observed in C) and D) correspond to \(\beta_1,4\)- and \(\beta_1,3\)-Gal linked isomers. See Figure 3 for nomenclature.

Supplementary figure 3. Supporting evidence for high antenna CBG N-glycans and the likely presence of \(\beta_1,3\)-linked Gal. A) CBG N-glycans were analysed using LC-PGC-ESI-IT-MS/MS without any treatment prior to release (top BPC), with broad specificity neuraminidase treatment prior to release (middle BPC), and with combined neuraminidase and \(\beta_1,4\)-specific galactosidase incubation prior to release (bottom BPC). The shift of analyte retention times clearly shows modulation of the N-glycan structures upon enzyme treatment. B) Summed MS full scans of the eluting area of the neuraminidase/\(\beta_1,4\)-specific galactosidase treated N-glycans. Fully desialylated and degalactosylated N-glycan structures i.e. bi-, tri-, tetra- and penta-antennary structures were present proving high antenna formation. A number of mono-
and di-galactosylated structures were still present. Based on the MS peak height, the non-\(\beta 1,4\)-linkage was found to be present for approximately 10-15% of all GlcNAc-Gal linkages (calculation not shown).

C) Upon fragmentation using CID MS/MS the Gal residues were confirmed to be located in the non-reducing terminal and ions formed from cross-ring cleavages strongly indicated the existence of \(\beta 1,3\)-linked Gal residues. Together, this confirms that CBG \(N\)-glycans are predominantly of the highly branched form as oppose to structures having repeating LacNAc units.

**Supplementary figure 4.** Differential accessibility to the glycosylation sites of human CBG. The glycosylation sites 2-5 of CBG were mapped on the available crystal structure of human CBG complexed with cortisol (PDB: 2VDY) (4) and visualised from two arbitrary angles (A and B) to illustrate the difference in site accessibility. Site 2 (Asn\(^{74}\)) and 3 (Asn\(^{154}\)) are exposed agreeing with the high degree of fucosylation and branching of the \(N\)-glycans observed from these sites (See glycoprofiling in Table 1 and Table 2), whereas site 4 (Asn\(^{238}\)) and 5 (Asn\(^{308}\)) are more buried and therefore less processed in terms of branching and core-fucosylation. To obtain the given crystal structure, human CBG was expressed recombinantly without Site 1 and 6. The relative accessible surface areas for the glycosylation sites 2-5 were calculated using NACCESS (40,41). In the program we used a probe radius size of 5Å (default 1.4 Å, radius as water) to roll around the atomic surface of the protein to calculate atomic solvent accessible areas; NACCESS uses those Van der Waals' radii derived by Chothia (42).

**Supplementary figure 5.** Location of CBG glycosylation site 4 (Asn\(^{238}\)). Glycosylation site 4 was mapped on the available crystal structure of human CBG complexed with cortisol (PDB: 2VDY) (4). This site was located near the entrance to the steroid binding site and conjugated \(N\)-glycans can thus be involved in the regulation of the accessibility of steroid to the site.
Supplementary figure 2

A. CBG N-glycan

B. CBG N-glycan

C. CBG N-glycan

D. CBG N-glycan

XIC (m/z 1111.3)

XIC (m/z 820.3)

XIC (m/z 1439.3)

XIC (m/z 1002.8)

+ α2,3-specific neuraminidase

+ α2,3-specific neuraminidase

+ α2,3-specific neuraminidase

+ α2,3-specific neuraminidase
Supplementary figure 4

Site 2 (Asn$^{74}$) – Rel. Accessibility: 1.00

Site 3 (Asn$^{154}$) – Rel. Accessibility: 0.80

Site 4 (Asn$^{238}$) – Rel. Accessibility: 0.28

Site 5 (Asn$^{308}$) – Rel. Accessibility: 0.23
Supplementary figure 5

Site 4 (Asn\textsuperscript{238})

Steroid binding site
(Cortisol bound)