Glycosylation of Human Corticosteroid-Binding Globulin. Differential Processing and Significance of Carbohydrate Chains at Individual Sites†

George V. Avvakumov* and Geoffrey L. Hammond‡

MRC Group in Fetal and Neonatal Health and Development and Departments of Obstetrics and Gynecology, Biochemistry, and Oncology, University of Western Ontario, London, Ontario N6A 4L6, Canada

Received December 20, 1993; Revised Manuscript Received February 23, 1994®

ABSTRACT: Human corticosteroid-binding globulin (CBG) comprises 383 amino acids and six consensus sites for attachment of *N*-acetyllactosamine-type oligosaccharides. To study the extent of addition and processing of individual carbohydrate chains, we expressed CBG mutants, each containing only one of the six possible glycosylation sites, in Chinese hamster ovary cells and examined their electrophoretic, immunochemical, and lectin-binding properties. This indicated that Asn^{9}, Asn^{308}, and Asn^{347} are partially glycosylated and that oligosaccharides attached to Asn^{9}, Asn^{238}, Asn^{308}, and Asn^{347} are predominantly biantennary, while more branched (most likely, triantennary) oligosaccharides are preferentially linked to Asn^{74} and Asn^{154}. Only one of the biantennary chains (attached to Asn^{9}) contains significant amounts of fucose. These data indicate that oligosaccharide processing is site-specific, and analyses of three other mutants, in which an additional glycosylation site was preserved, demonstrated that the processing of individual oligosaccharides occurs independently. Thus, the glycosylation of recombinant CBG appears to resemble that of natural human CBG. As we have previously found, glycosylation at Asn^{238} is essential for the production of CBG with steroid-binding activity, but when the mutant containing only one oligosaccharide at this position was enzymatically deglycosylated, its steroid-binding activity was unaltered. This suggests that interaction between this carbohydrate chain and the polypeptide is necessary for the folding and creation of the steroid-binding site only during CBG biosynthesis.

Glycosylation provides proteins with an additional level of structural complexity that may influence their biosynthesis, secretion, and biological activity (Rademacher et al., 1988; Paulson, 1989; Drickamer, 1991; Cumming, 1992; Hartree & Renwick, 1992). The requirements for specific carbohydrate structures at particular locations are as yet poorly defined with respect to these events, and plasma corticosteroid-binding globulin (CBG[1]) provides a useful experimental model because, in addition to binding natural glucocorticoids and progesterone with high affinity (Hammond, 1990), it interacts with the plasma membranes of specific cell types (Avvakumov, 1991; Hryb et al., 1986). Furthermore, as demonstrated for the glycoprotein hormones (Hartree & Renwick, 1992), the plasma membrane binding of CBG appears to depend on its carbohydrate composition (Avvakumov, 1991), which may vary under different physiological conditions (Strel'chyonok et al., 1984). The types of oligosaccharides associated with CBG may also influence its compartmentalization within the organism (Seralini et al., 1989; Avvakumov & Strel'chyonok, 1987), and changes in its glycosylation may be biologically important during development (Berdusco et al., 1993).

When isolated from human serum, CBG contains 5.1 mol of *N*-acetyllactosamine-type oligosaccharides/(mol of glycoprotein) (Strel'chyonok et al., 1982). However, the cDNA-deduced sequence of human CBG contains six consensus sites for *N*-glycosylation (Hammond et al., 1987), and expression of recombinant human CBG in Chinese hamster ovary (CHO) cells has shown that all of them may be utilized (Avvakumov et al., 1993). Information about the types of sugar chains attached to specific consensus sites is limited, but CBG purified from human serum comprises biantennary and triantennary oligosaccharides in a 3:2 molar ratio (Strel'chyonok et al., 1982). There is also evidence that the site closest to the carboxy terminus of the molecule is partially utilized (Hammond et al., 1990) and that specific processing of oligosaccharides occurs at two of the other five sites (Avvakumov et al., 1993).

We have recently demonstrated that glycosylation at a phylogenetically conserved consensus site (Asn^{238}-Gly^{239}-Thr^{240}) is an absolute requirement for the production of human CBG with appropriate steroid-binding activity (Avvakumov et al., 1993). However, it is not known whether the carbohydrate chain at this position actively participates in steroid binding, whether it influences the conformational stability of the protein, or whether it affects the folding of the nascent polypeptide that ultimately leads to formation of the steroid-binding site. The first possibility is unlikely because almost complete removal of carbohydrates from human CBG with a mixture of exoglycosidases has no influence on steroid binding (Mickelson et al., 1982). The latter two possibilities both imply that an intramolecular carbohydrate–polypeptide interaction occurs, and this is supported by the limited processing of the oligosaccharide attached to Asn^{238} (Avvakumov et al., 1993).

A panel of CBG mutants, each containing only one of the six consensus sites for *N*-glycosylation, has therefore been constructed to determine how extensively these sites are utilized and whether processing of the sugar chains is site-specific. Additional consensus sites for glycosylation were also preserved in several other mutants to examine whether processing at

† This work was supported by grants from the Medical Research Council (MRC) of Canada and Allelix Biopharmaceuticals Inc.

* Address correspondence to this author at London Regional Cancer Centre, 790 Commissioners Road East, London, Ontario N6A 4L6, Canada. Telephone: 519-685-8617. Fax: 519-685-8616.

‡ G. L. H. is an Ontario Cancer Treatment and Research Foundation Scholar.

® Abstract published in *Advance ACS Abstracts,* April 1, 1994.

[1] Abbreviations: CBG, corticosteroid-binding globulin; CHO cells, Chinese hamster ovary cells; RIA, radioimmunoassay; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Con A, concanavalin A; LCA, lectin from *Lens culinaris*.

0006-2960/94/0433-5759$04.50/0

Table 1: Location of *N*-Glycosylation Sites in CBG Mutants and Their Concentrations in Culture Medium[a]

protein	location of *N*-glycosylation consensus sites	CBG concn in culture medium (nM)	K_a (M^{-1})
	ApaI *SalI* *StuI*		
wild type	I II III IV V VI N—NMS NLT NKT NGT NFS NLT—C	1.2	1.3×10^9
I+	N—NLA QKT QGT QFS QLT—C	0.1	*b*
II+	N—QMS QKT QGT QFS QLT—C	0.8	*b*
II/III+	N—QMS QGT QFS QLT—C	0.9	*b*
III+	N—QMS NLA QGT QFS QLT—C	0.1	*b*
II/IV+	N—QMS QKT QFS QLT—C	0.8	2.1×10^8
IV+	N—QMS NLA QKT QFS QLT—C	0.9	1.3×10^8
V+	N—QMS NLA QKT QGT QLT—C	0.1	*b*
V/VI+	N—QMS NLA QKT QGT—C	0.5	*b*
VI+	N—QMS NLA QKT QGT QFS—C	0.1	*b*
0	N—QMS QLT QKT QGT QFS QLT—C	<0.05	*b*

[a] Relative positions and sequences of consensus sites for *N*-glycosylation (I–VI) are shown for wild-type CBG; sites I, II, III, IV, V, and VI correspond to Asn residues at positions 9, 74, 154, 238, 308, and 347 in the CBG polypeptide, respectively. Only the sequences of altered consensus sites are shown for the mutant proteins. Relative positions of unique restriction sites in the CBG cDNA, used for the construction of cDNAs with multiple mutations, are indicated. The amounts of CBG in the culture media were assayed by RIA (mean of two determinations). Affinity constants were determined as described in the text after adjustment of the CBG concentration in the media to 0.8–1.3 nM by ultrafiltration. [b] No detectable binding activity.

specific sites is influenced by the presence of a second carbohydrate chain. We have also assessed the consequences of enzymatically deglycosylating a CBG mutant containing only one carbohydrate chain attached to Asn^{238}, with respect to its steroid-binding properties, and have tried to define the interaction(s) that may occur between this chain and amino acid residues in the CBG molecule.

EXPERIMENTAL PROCEDURES

Construction and Expression of Mutant CBG cDNAs. The cDNAs encoding the wild-type human CBG precursor and four of the mutants listed in Table 1 (II+, II/IV+, IV+, and 0) were constructed within the *Hin*dIII/*Xba*I sites of pSelect-1 (Promega), as described previously (Avvakumov et al., 1993). Additional mutant cDNAs were obtained by exchanging appropriate DNA fragments generated from these and other existing (Avvakumov et al., 1993) mutants by restriction endonuclease digestion at unique *Apa*I, *Sal*I, and *Stu*I sites (Table 1). When necessary, additional mutations were introduced by site-directed mutagenesis of the cDNAs in pSelect according to the protocol provided by Promega. Oligonucleotides (20–26-mers) used for mutagenesis were synthesized by the Molecular Biology Core Facility of the MRC Group in Fetal and Neonatal Health and Development. Mutated cDNAs were sequenced (Sanger et al., 1977) to confirm that only targeted mutations had occurred and then were subcloned into a *Hin*dIII/*Xba*I-digested pRc/CMV (Invitrogen) for expression in Chinese hamster ovary (CHO pro$^-$, wild type) cells (Avvakumov et al., 1993). After selection in the presence of Geneticin (Gibco/BRL), resistant cells were expanded to near confluence, washed twice with PBS, and then cultured for 2 days in Dulbecco's modified Eagle medium containing 100 nM cortisol.

CBG Assays. The CBG concentrations in culture media were determined using a radioimmunoassay (RIA), as previously described (Avvakumov et al., 1993). The steroid-binding capacity of CBG was measured by saturation analysis using [^{3}H]cortisol (55.8 Ci/mmol; DuPont) as labeled ligand and dextran-coated charcoal to separate bound and free steroid (Hammond & Lähteenmäki, 1983). The affinities of CBG mutants for cortisol were measured by Scatchard analysis (Scatchard, 1949; Hammond & Lähteenmäki, 1983) after appropriate concentration of culture media using Centricon microconcentrators (Amicon); the association constants (K_a) reported represent mean values of two experiments.

Western Blots. Polyacrylamide gel electrophoresis (4% stacking and 10% resolving gels) in the presence of sodium dodecyl sulfate (SDS–PAGE) was performed as previously described (Laemmli, 1970), and proteins were transferred to Hybond-ECL (Amersham) membranes by electroblotting (Towbin et al., 1979). The blots were blocked and incubated with a rabbit antiserum raised against human CBG (1:500 diluted), and immunoreactive proteins were visualized using the ECL Western blotting analysis system (Amersham).

Lectin-Affinity Chromatography. For chromatography on immobilized concanavalin A (Con A), samples (0.7–1 mL) of culture medium were adjusted by ultrafiltration to contain similar concentrations (4–5 nM) of immunoreactive CBG and applied onto 3-mL Con A–Sepharose (Pharmacia) columns preequilibrated in lectin column buffer (10 mM Tris, pH 7.4, 500 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$ containing 0.01% bovine serum albumin), followed by 0.5 mL of the

same buffer. After a 15-min delay to allow glycoproteins to interact with the lectin, elution was performed with 9 mL of the lectin column buffer followed by 15 mL of the same buffer containing 20 mM methyl α-D-mannopyranoside. This procedure resulted in complete elution of the applied glycoproteins from the affinity resin; subsequent washing of the column with a buffer containing 200 mM methyl α-D-mannopyranoside gave no immunoreactive material in the eluate. Fractions of 1 mL were collected. Chromatography on immobilized lectin from *Lens culinaris* (LCA) was performed essentially as above using a 0.5-mL column packed with LCA covalently attached to 4% beaded agarose (Sigma). The sample volume was 0.5 mL, and two 1.5-mL fractions were collected: the first was eluted with the lectin column buffer, and the second fraction was collected after elution with the same buffer containing 100 mM methyl α-D-mannopyranoside. In both cases, chromatographic fractions were analyzed by RIA (Avvakumov et al., 1993), and the amount of CBG recovered from the column after elution with the buffer containing 20 mM (Con A+) or 100 mM (LCA+) mannoside was expressed as a percentage of the total amount of CBG in all chromatographic fractions.

Enzymatic Desialylation and Deglycosylation. Culture media were concentrated by ultrafiltration to yield a CBG concentration of 4–6 nM. During this procedure, the buffer was changed to 0.1 M sodium phosphate, pH 7.2, containing 25 mM EDTA and 0.5 μM cortisol to stabilize the proteins, and the samples were digested for 16 h at 37 °C with neuraminidase (EC 3.2.1.18) from *Clostridium perfringes* (Boehringer Mannheim). For desialylation and complete removal of the carbohydrate chains attached to mutant IV+, digestion was performed for 72 h at 37 °C with neuraminidase alone and with a combination of neuraminidase and *N*-glycosidase F (EC 3.5.1.52; Boehringer Mannheim), respectively. Reaction products were analyzed by SDS–PAGE/Western blotting, and the steroid binding affinity constants of desialylated and deglycosylated mutant IV+ were determined as described above.

RESULTS

Influence of Specific Carbohydrate Chains on Recombinant CBG Production. Table 1 illustrates the location of consensus sites for *N*-glycosylation in the wild-type and mutant forms of human CBG expressed in CHO cells, together with the concentrations of immunoreactive CBG in the culture media. Dose–response curves for all CBG mutants were indistinguishable from that obtained for wild-type human CBG in a standard RIA (data not shown). This indicated that site-directed mutagenesis did not alter the immunochemical properties of CBG (and, consequently, its overall conformation) and validated the use of RIA for the measurement of the concentrations of the mutants. The mutants containing only consensus site II or IV were secreted at levels close to that of wild-type CBG, while the secretion of other mutants containing only one carbohydrate chain was markedly reduced. However, the addition of a second chain to these mutants clearly increased the amount of CBG in the culture media (Table 1). Although all CBG mutants were readily detectable in unconcentrated culture medium, those mutants lacking consensus site IV displayed no steroid-binding activity, even after concentration of the medium (Table 1).

Specificity of Oligosaccharide Addition at Individual Sites. Aliquots of culture media containing the CBG mutants listed in Table 1 were analyzed by Western blotting (Figure 1 A). Differences in the electrophoretic mobilities of mutants

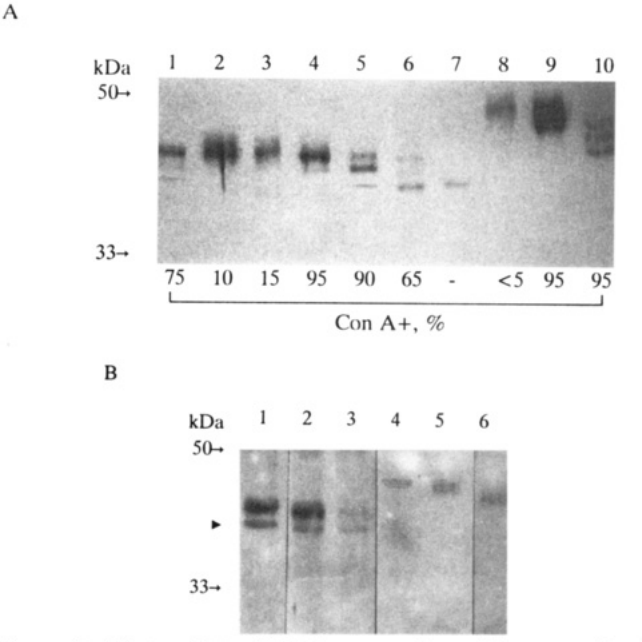

FIGURE 1: Western blots of CBG mutants. Recombinant proteins in the culture medium (about 1 ng/well) were separated electrophoretically in a 10% polyacrylamide gel in the presence of SDS, electroblotted onto a Hybond-ECL nitrocellulose membrane, and visualized as described under Experimental Procedures. Arrows indicate positions of prestained protein standards. (A) Lanes 1–6, mutants with one consensus site for *N*-glycosylation (mutants I+ to VI+, respectively, in Table 1); lane 7, unglycosylated CBG (mutant 0 in Table 1); lanes 8–10, mutants containing two glycosylation sites (mutants II/III+, II/IV+, and V/VI+, respectively, in Table 1). The relative amounts (Con A+, %) of material retarded and eluted with 20 mM methyl α-mannopyranoside during Con A chromatography of the corresponding mutants are indicated below the lanes. (B) Desialylated mutants I+, V+, VI+, II/III+, II/IV+, and V/VI+ (lanes 1–6, respectively). The arrowhead indicates the position of unglycosylated CBG.

containing only one consensus site for *N*-glycosylation (lanes 1–6) reflect variations in utilization and differential processing of their oligosaccharide chains. In the lanes containing mutants I+, V+, and VI+, an immunoreactive band is visible that has an electrophoretic mobility similar to that of unglycosylated CBG (mutant 0, Table 1) and therefore likely corresponds to unglycosylated CBG. This indicates partial utilization of the consensus sites closest to the amino and carboxy termini of the CBG polypeptide. The lower electrophoretic mobility of mutants II+ and III+, as compared to the other mutants with only one glycosylation site, corresponds to the increase in M_r of about 1 kDa. Taken together with the relative amounts of Con A-reactive glycoforms associated with each mutant (Figure 1A), this implies the presence of biantennary oligosaccharides at sites I, IV, V, and VI and more branched (most likely, triantennary) carbohydrate chains at sites II and III. Complete elution of Con A-reactive glycoforms from the affinity column at a low concentration of mannoside in the elution buffer indicates the absence of high-mannose and hybrid-type oligosaccharides. The mutants with one glycosylation site show electrophoretic heterogeneity, and, in some instances, these can be resolved into several bands (see, e.g., lanes 1, 5, and 6 in Figure 1A). Although this level of electrophoretic resolution was difficult to reproduce, the microheterogeneity observed probably reflects a variable degree of sialylation of the antennae. Reduction of the number of electrophoretic bands observed for individual mutants after treatment with neuraminidase (Figure 1B) supports this assumption.

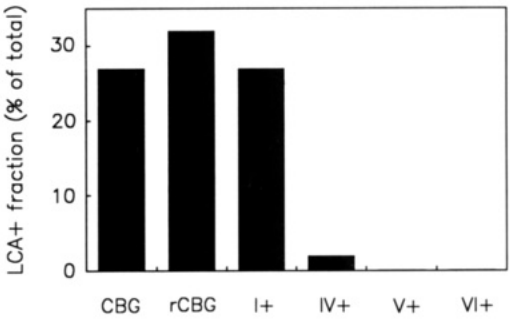

FIGURE 2: Fucosylation of biantennary oligosaccharides in CBG. Samples of CBG purified from human serum, recombinant wild-type CBG (rCBG), and mutants containing only one biantennary carbohydrate chain (I+, IV+, V+, and VI+) were applied onto an LCA–agarose column. Relative amounts of CBG retarded by the column (LCA+ fraction) are expressed as a percentage of the total immunoreactive CBG eluted from the column.

Oligosaccharide Processing in the Presence of Two N-Glycosylation Consensus Sites. The electrophoretic mobilities of mutants containing two sites for *N*-glycosylation (Figure 1A, lanes 8–10) can be satisfactorily explained by a combination of carbohydrate chains similar to those associated with mutants bearing the corresponding consensus sites alone. For instance, mutant II/III+ migrates as a diffuse electrophoretic band with the lowest mobility (M_r = 46–48 kDa). Moreover, as observed for the corresponding mutants with single sugar chains at these sites, mutants II+ and III+ (Table 1), Con A chromatography showed the virtual absence of biantennary sugar chains in mutant II/III+; i.e., less than 5% of this mutant was retarded by the column. Similarly, mutant V/VI+ is characterized by a set of three bands with M_r's ranging from 42 to 45 kDa, the most mobile of which corresponds to a CBG form with only one biantennary sugar chain, and this is in line with a partial utilization of site VI and the presence of only biantennary chains in both mutant V+ and mutant VI+. Mutant II/IV+ exhibited an intermediate mobility (M_r = 45–47 kDa) when compared to the two other mutants. This can be explained by the combination of a biantennary sugar chain, which allows almost complete retardation during Con A chromatography (as in mutant IV+), and a more branched chain (as in mutant II+) that accounts for its slower electrophoretic mobility when compared to mutant V/VI+. The greater electrophoretic heterogeneity of all three of these mutants is also consistent with the presence of two carbohydrate structures that both contain variable amounts of sialic acid, and this is supported by the fact that treatment with neuraminidase reduced their electrophoretic heterogeneity (Figure 1B). Collectively, these data suggest that the processing of these carbohydrate chains occurs independently and in a site-specific manner.

Fucosylation of Biantennary Sugar Chains. Figure 2 presents the results of LCA chromatography of CBG purified from human serum, recombinant wild-type CBG, and mutants containing only one biantennary oligosaccharide chain. Based on the specificity of this lectin, which recognizes only fucosylated biantennary chains of the *N*-acetyllactosamine type (Yamamoto et al., 1993), the percentage of immunoreactive CBG retarded by the column reflects the proportion of CBG molecules containing this type of oligosaccharide. These data therefore show that attachment of fucose to biantennary oligosaccharides in recombinant CBG is similar to that in CBG derived from human serum and is almost exclusively limited to the carbohydrate chain linked to glycosylation site I.

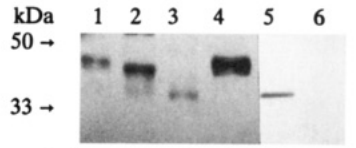

FIGURE 3: Enzymatic deglycosylation of the CBG mutant containing only the carbohydrate chain at Asn^{238}: SDS–PAGE/Western blot of desialylated (lane 2) and deglycosylated (lane 3) mutant IV+ (Table 1) as compared with untreated mutant IV+ (lane 4) and non-glycosylated CBG (lane 5; mutant 0, Table 1). Controls: mutant IV+ incubated in the absence of enzymes (lane 1) and conditioned culture medium from untransfected CHO cells (lane 6).

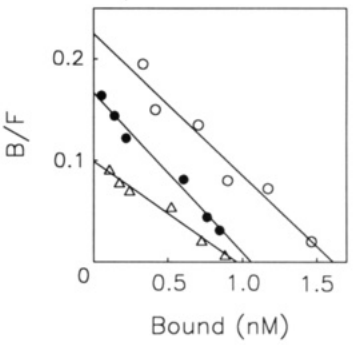

FIGURE 4: Scatchard plots of cortisol binding to enzymatically modified and intact mutant IV+. The binding activities of untreated (○), desialylated (●), and deglycosylated (△) forms of mutant IV+ (see Figure 3) were assessed by Scatchard analysis with [^{3}H]cortisol as a labeled ligand.

Steroid Binding of Mutant IV+ after Enzymatic Deglycosylation. Preliminary experiments showed that *N*-glycosidase F did not effectively remove carbohydrates from the mutant containing a single carbohydrate chain at Asn^{238} (mutant IV+, Table 1), when used alone under the non-denaturing conditions that are necessary to maintain the steroid-binding activity of CBG (Chan & Slaunwhite, 1977). Therefore, a combination of *N*-glycosidase F and neuraminidase was used to achieve complete deglycosylation of this mutant. As shown in Figure 3, this resulted in a product with the same electrophoretic mobility as unglycosylated CBG (mutant 0), which is highly indicative of the complete removal of carbohydrates. Scatchard analysis revealed that neither desialylation nor complete deglycosylation influenced the affinity of this CBG mutant for cortisol (Figure 4). A decrease in the cortisol-binding capacities of the desialylated and deglycosylated forms of mutant IV+ may be explained by their partial denaturation during prolonged incubation at 37 °C.

Mutation of Trp Residues in Mutant IV+. In order to examine the possible involvement of Trp residues in the interaction with the oligosaccharide chain attached to Asn^{238}, the codons corresponding to the four Trp residues in the CBG mutant, containing only one consensus site for glycosylation at this residue, were mutated individually to encode Phe at these positions. No differences were observed in the electrophoretic mobilities of the Trp → Phe mutants at positions 141, 185, and 371 (Figure 5), and their behavior during Con A chromatography was indistinguishable from that of mutant IV+ (data not shown). These mutants were easily detectable in culture media by RIA, but their concentrations were lower than that of mutant IV+ (Figure 5). By contrast, no immunoreactive CBG was detected in the medium from cells transfected with the cDNA encoding mutant IV+ in which Trp^{266} was substituted by Phe (Figure 5). Furthermore, this mutant was virtually undetectable by Western blotting, even after a 10-fold concentration of the medium (Figure 5), and

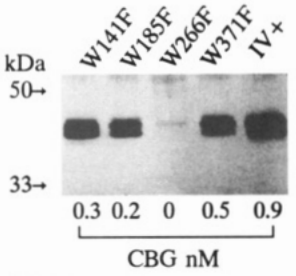

FIGURE 5: Effect of the Trp → Phe mutations on the secretion and electrophoretic mobility of mutant IV+. Tryptophan residues were individually substituted with phenylalanine in the CBG mutant containing only the phylogenetically conserved carbohydrate chain (mutant IV+, Table 1), as indicated at the top, and the proteins were analyzed by SDS–PAGE and Western blotting. The concentrations of immunoreactive CBG in the corresponding culture media are indicated below the lanes.

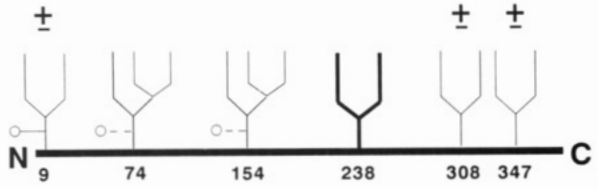

FIGURE 6: Glycosylation of human CBG. Preferential locations of biantennary and triantennary oligosaccharide chains are shown; positions of the corresponding Asn residues are indicated below the bar representing the CBG polypeptide. Microheterogeneity with respect to partial utilization of three of the consensus sites for *N*-glycosylation is depicted by the symbol "±". Fucose residues are shown as O; putative attachment of fucose to triantennary chains is shown by dashed lines. The carbohydrate chain that is essential for the proper folding of CBG and the acquisition of its steroid-binding activity is shown in bold.

similar results were obtained in three separate transfection experiments.

DISCUSSION

The presence of carbohydrate chains may protect glycoproteins from proteolytic degradation, improve their solubility, and contribute to their conformational stability (Rademacher et al., 1988). The variety of different monosaccharide units, the possible differences in the linkages between them, and their potential for branching provides oligosaccharides with an enormous structural diversity compared to other biopolymers that may contribute to many cellular and molecular recognition processes (Montreuil, 1984; Cumming, 1992; Kobata, 1993). Carbohydrate structures may also influence the folding of newly synthesized proteins (Kern et al., 1992; Ruddon et al., 1987), which in turn is necessary for the proteins' intracellular sorting, secretion, and acquisition of specific biological functions (Cumming, 1992). The specificity of oligosaccharide processing at defined locations within polypeptides is therefore obviously an important issue, and we have used CBG to examine this because the carbohydrates attached to this protein appear to influence its production and biological activity at several levels. The creation of glycoproteins with altered oligosaccharide content by site-directed mutagenesis provides a unique opportunity to study both the structure and the functional significance of individual carbohydrate chains. While it is true that glycosylation of recombinant glycoproteins may be influenced by the host cell type and the growth conditions (Patel et al., 1992; Mackiewicz et al., 1993), detailed analyses of numerous recombinant glycoproteins expressed in mammalian cells, including CHO cells, have revealed striking structural similarities between their carbohydrate moieties and those of their natural counterparts (Hard et al., 1990; Tsuda et al., 1988; Takeuchi et al., 1988; Sasaki et al., 1987; Nimtz et al., 1993).

We have previously found that recombinant human CBG expressed in CHO cells contains biantennary and multiantennary oligosaccharides of the *N*-acetyllactosamine type (Avvakumov et al., 1993), and a systematic analysis of CBG mutants containing only one consensus site has now enabled us to demonstrate that the sites closest to the amino and carboxy termini of the polypeptide are only partially utilized (Figure 6). This is in line with a weak signal corresponding to the phenylthiohydantoin derivative of unglycosylated Asn at the ninth step of Edman degradation of CBG purified from human serum (our unpublished observation) and the size heterogeneity of the fragment (Thr^{345}–Val^{383}) cleaved from human CBG by neutrophil elastase (Hammond et al., 1990). Previous analyses of two CBG mutants containing only one potential site for *N*-glycosylation (II or IV) also suggested that the processing of oligosaccharide chains in human CBG is site-specific (Avvakumov et al., 1993). Our present data confirm this and demonstrate that processing of carbohydrate chains at sites I, IV, V, and VI is largely limited to biantennary oligosaccharides, while those attached to sites II and III are further processed to form multibranched (more likely, triantennary) glycans. Analyses of mutants containing two glycosylation sites have revealed little or no interdependence in the processing of carbohydrate chains, and it is therefore likely that the oligosaccharides associated with these mutants closely resemble those present in recombinant wild-type human CBG. Furthermore, when the CBG mutants with only a single glycosylation site are considered together, with respect to the utilization of individual sites and the structures of their associated carbohydrate chains, they yield an estimate of oligosaccharide content and composition that closely approximates those determined by direct analysis of purified human CBG, i.e., 5.1 mol of biantennary and triantennary oligosaccharides in a 3:2 ratio/(mol of glycoprotein) (Strel'chyonok et al., 1982).

Attachment of fucose to oligosaccharides of the *N*-acetyllactosamine type may significantly alter their conformation and, consequently, their potential as determinants of molecular recognition (Montreuil, 1984). Different glycoproteins contain variable amounts of fucose, and fucosylation is certainly not a random or nonspecific process. For example, another hepatic protein closely related in structure to CBG, thyroxine-binding globulin, contains no fucosylated oligosaccharides (Avvakumov & Strel'chyonok, 1985). We have now shown that recombinant wild-type CBG and the mutant with only one sugar chain attached to the site closest to its amino terminus (site I) are both indistinguishable from serum-derived CBG with respect to their ability to interact with a lectin from *Lens culinaris*. This not only demonstrates that fucosylation of biantennary oligosaccharides in human CBG is site-specific, but again provides evidence that the glycosylation of human CBG expressed in CHO cells closely resembles that of its natural counterpart. However, it has been demonstrated that CBG from human serum contains 1.2 mol of fucose/(mol of glycoprotein) (Strel'chyonok et al., 1982), and because only about 30% of CBG molecules (irrespective of their source) contain a fucosylated biantennary chain, we conclude that one or both of the triantennary chains attached to sites II and III represent the primary site(s) of fucosylation.

Among the panel of six human CBG mutants with one carbohydrate chain, only the mutant containing a nonfucosylated biantennary chain at Asn^{238} (mutant IV+) has

steroid-binding activity. This confirms our previous observation that this carbohydrate chain is essential for the creation of the steroid-binding site, and explains why it is so conserved between species (Avvakumov et al., 1993). We have now demonstrated that its removal does not influence the steroid-binding activity of mutant IV+. Thus, it appears that this carbohydrate chain is necessary for the CBG molecule to acquire its appropriate structure, but once this has occurred, it no longer influences the conformation of the steroid-binding site, which must therefore be maintained solely by the overall tertiary structure of the polypeptide.

The obvious importance of glycosylation at Asn^{238} suggests that a carbohydrate chain at this position interacts with the nascent polypeptide to dictate the folding of the glycoprotein during biosynthesis. This assumption is supported by the limited processing of the carbohydrate chain and by evidence that a carbohydrate chain at an analogous position in α_1-antitrypsin (Hammond et al., 1987) probably interacts with tryptophan (Powell & Pain, 1992). This amino acid has also been found in the active sites of carbohydrate-transforming enzymes (Poole et al., 1993) and carbohydrate-binding proteins (Spurlino et al., 1992), and we therefore focused our attention on the tryptophan residues in CBG as candidates for interaction with the conserved carbohydrate chain at Asn^{238}. A comparison of the cDNA-deduced primary structures of CBGs from various species (Hammond et al., 1991; Scrocchi et al., 1993; Berdusco et al., 1993) showed that all four Trp residues in human CBG are invariably conserved, and we employed site-directed mutagenesis to individually substitute Trp residues with Phe in mutant IV+. While the Trp → Phe mutations at positions 141, 185, and 371 resulted in the production of CBG molecules with electrophoretic and lectin-binding properties similar to those of mutant IV+, the mutation at position 266 completely abolished secretion of the glycoprotein. In a separate study (Avvakumov & Hammond, 1994) we fintroduced a Trp^{266} → Phe mutation in wild-type human CBG, and this resulted in only a 54% decrease in the production of the recombinant glycoprotein and an 8-fold decrease in its affinity for cortisol. Therefore, while an interaction between the oligosaccharide attached to Asn^{238} and this tryptophan may contribute to the proper folding of nascent CBG molecules, the presence of other carbohydrate chains may partially compensate for this. Furthermore, Trp^{266} is obviously not the only amino acid that could participate in such an interaction.

In conclusion, the processing of oligosaccharides in human CBG appears to be site-specific, and the carbohydrate composition of CBG expressed in CHO cells closely resembles that of the natural protein isolated from human blood (Figure 6). Although this suggests that glycosylation events are controlled to some extent by the structure of the polypeptide, it is important to note that a natural variant of CBG with only triantennary oligosaccharides exists in human pregnancy serum (Avvakumov & Strel'chyonok, 1987). Therefore, specific factors that influence glycosylation events at the cellular level (Rademacher et al., 1988) may override any constraints imposed by the polypeptide structure. A carbohydrate chain that influences the folding of the protein and the formation of the steroid-binding site during biosynthesis has been identified as a non-fucosylated biantennary oligosaccharide, but this particular structure is not critical because the pregnancy-associated variant containing only triantennary oligosaccharides binds steroids normally (Avvakumov & Strel'chyonok, 1987), and human CBG with appropriate steroid-binding activity has been produced in insect cells (Ghose-Dastidar et al., 1991) that only synthesize oligosaccharides of high-mannose type (Chen & Bahl, 1991). Furthermore, since this chain can be removed without affecting steroid binding, carbohydrate–polypeptide interactions appear to be responsible for creating the steroid-binding site during early stages of CBG biosynthesis but play no role in maintaining its integrity. It is not clear how this carbohydrate chain interacts with the polypeptide, but contacts with Trp^{266} seem to be important for biosynthesis. However, resolution of this issue must await the development of a technique that will enable definition of this type of transient carbohydrate–protein interaction during cotranslational glycosylation and folding of the nascent glycoprotein. Although the apparent specificity of glycosylation at sites II, III, and IV might imply that they are functionally important, it cannot be excluded that partial utilization of sites I, V, and VI results in glycoforms with specialized biological activities. Given the multifunctional properties of CBG, it will therefore be important to utilize CBG mutants similar to those described here to assess their biological activities *in vivo*.

ACKNOWLEDGMENT

We would like to thank Gail Howard and Denise Hynes for secretarial assistance, Wayne Bocchinfuso for his helpful comments, and Susan Warmels-Rodenhiser and Allen Grolla for technical assistance.

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