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Expression of Biologically Active Human Corticosteroid Binding Globulin by Insect Cells: Acquisition of Function Requires Glycosylation and Transport

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Expression of biologically active human corticosteroid binding globulin by insect cells: Acquisition of function requires glycosylation and transport

(baculovirus-mediated expression/*in vitro* translation/protein folding)

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ABSTRACT **Human corticosteroid binding globulin (hCBG) is a 50- to 55-kDa serum glycoprotein that binds cortisol and progesterone with high affinity. To map the steroid-binding domain and to investigate the folding pathways of hCBG, we have established an expression system based on infection of insect cells with a recombinant baculovirus encoding hCBG. Infected *Spodoptera frugiperda* (Sf9) cells secrete immunoreactive hCBG at high levels (16–24 pmol per 10^6 cells per 40 h), and the recombinant protein binds cortisol with an affinity and specificity equivalent to that of human serum-derived hCBG. Thus, this system has the potential to provide large amounts of wild-type and mutant hCBGs for physical-chemical analysis. Cotranslational asparagine-linked glycosylation is essential for acquisition of steroid-binding capability, as shown by the lack of cortisol-binding activity of unglycosylated hCBG secreted in the presence of tunicamycin. Golgi-associated oligosaccharide processing, however, is not required for activity, as demonstrated by the endoglycosidase H susceptibility of the fully active, secreted glycoprotein. Comparison of the steroid-binding properties of intracellular and secreted hCBG with that synthesized *in vitro* in the rabbit reticulocyte lysate system suggests that this protein undergoes a maturation process during transport through the secretory pathway. This system will be useful for identifying the molecular determinants of biological function in hCBG.**

The steroid-binding globulins are excellent model systems for studying how protein structure and conformational dynamics determine function. The diverse biological activities proposed for these serum proteins suggest that they contain multiple functional domains. Their high-affinity ligand binding provides a sensitive indicator of proper tertiary structure, facilitating the analysis of protein folding *in vivo* and *in vitro*. Furthermore, the postsynthetic transport of these glycoproteins through endoplasmic reticulum (ER) and Golgi compartments raises the possibility of identifying intracellular folding intermediates. To incorporate biophysical and cell biological approaches, we have established a system for high-level expression of human corticosteroid binding globulin (hCBG), and we report here our first correlations of structure and function in this polypeptide.

In humans, 80–90% of the cortisol present in serum is bound with high affinity and specificity to hCBG (1, 2). hCBG is an acidic glycoprotein synthesized primarily in liver and kidney, and it is secreted into the circulation as a 50- to 55-kDa monomer containing a single steroid-binding site (2). Its cDNA sequence predicts a 405-amino acid protein, which includes a 22-residue amino-terminal signal peptide and six consensus sites for asparagine-linked glycosylation; overall, hCBG shares significant homology with members of the serpin (serine protease inhibitor) family of protease inhibitors (3).

A generally accepted function of hCBG is the maintenance of an appropriate balance of free and protein-bound cortisol in serum (1, 2, 4, 5). It has recently been proposed that release of cortisol from hCBG following proteolytic cleavage by neutrophil-derived elastase provides a mechanism to target cortisol to sites of inflammation (6, 7). The observation of high-affinity cell-surface binding sites for hCBG (8, 9) suggests an additional role in cortisol delivery and uptake, or, alternatively, in a cortisol-regulated signal transduction pathway (10). To reconcile and understand these possible modes of action of hCBG will require the identification of steroid- and receptor-binding domains within this polypeptide and the delineation of specific features that regulate steroid binding and release.

At present we have limited knowledge of the structure of the steroid-binding site of hCBG, since a crystallographic description of this protein has not yet been achieved. The interaction of hCBG with cortisol protects one of its two cysteine residues from chemical modification (11) and also quenches the fluorescence of one or more of its four tryptophans (12). Notably, Cys-228 and the four tryptophans are conserved between human, rat, and rabbit CBG sequences (3, 13, 14), suggesting that they have functional significance; there have been no reports, however, of site-directed mutants in which steroid binding is altered.

To investigate how hCBG acquires steroid-binding activity, we have expressed the wild-type protein *in vitro*, in the reticulocyte lysate system, and *in vivo*, in insect cells infected with an hCBG recombinant baculovirus (15). Here we compare the properties of recombinant hCBG with those of native hCBG purified from human serum. We report that insect cells secrete high levels of hCBG that is fully active in cortisol binding. To our knowledge, this is the first demonstration that the cDNA identified by Hammond *et al.* (3) encodes authentic, functional hCBG. Using this system, we have determined that acquisition of binding activity requires core glycosylation in the ER and may also involve subsequent maturation steps.

METHODS

Cloning of CBG cDNA. CBG cDNA was isolated from a human liver cDNA library that had been cloned into the *Eco*RI site of λgt11 phage DNA (Clontech). Plaques containing CBG cDNA were identified by hybridization of immobilized phage to ^{32}P-labeled synthetic oligonucleotide probes derived from 5′ and 3′ regions of the hCBG cDNA sequence

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Abbreviations: hCBG, human corticosteroid binding globulin; ER, endoplasmic reticulum; S^+-, S^--, and GP-hCBG, signal peptide-containing, signal peptide-cleaved, and glycosylated forms of hCBG, respectively.

(4). The 5′ probe corresponded to nucleotides 25–49 (5′-TATACTGGACAATGCCACTCCTCCT-3′), and the 3′ probe corresponded to nucleotides 1371–1395 (5′-CAGACTTGTGTCTAACTTTAGGCAT-3′). Phage DNA was purified from plaques to which both probes hybridized. One isolate contained a 1.4-kilobase *Eco*RI insert whose restriction map was that predicted for full-length hCBG cDNA, and partial DNA sequencing confirmed the presence of the initiator ATG codon and 35 base pairs of 5′ sequence (4). This insert was amplified by cloning into *Eco*RI-digested pGEM1 (Promega), producing pG-CBG1, which served as a vector for *in vitro* transcription reactions (see below). The *Eco*RI insert was then cloned into the unique *Eco*RI site of the baculovirus-derived pVL-1393 vector (Invitrogen), placing hCBG expression under control of the baculovirus polyhedrin promoter. This recombinant plasmid was designated pVL-CBG1.

***In Vitro* Transcription and Translation.** CBG mRNA was synthesized *in vitro* using pG-CBG1 as template and T7 RNA polymerase, according to the manufacturer's protocols (Promega). The resulting mRNA was used to program cell-free protein synthesis in the rabbit reticulocyte lysate system (Promega); where indicated, reaction mixtures were supplemented with canine pancreatic microsomes prepared by the method of Shields and Blobel (16).

Production of Recombinant Baculovirus. A stock of recombinant baculovirus was produced by the method of Summer and Smith (15) using pVL-CBG1 DNA and AcMNPV (baculovirus) DNA for the mixed transfection of *Spodoptera frugiperda* (Sf9) cells. Recombinant baculoviruses were identified by hybridization with ^{32}P-labeled hCBG cDNA and purified by visual screening of plaque morphology (15). Viral titers were determined by plaque assay in Sf9 cells.

Infection and Metabolic Labeling. For expression of hCBG, Sf9 cells were infected with recombinant virus at a multiplicity of 10–25 plaque-forming units per cell. For radiolabeling, cells were incubated with medium lacking unlabeled methionine and containing [^{35}S]methionine at 100 μCi/ml (specific activity = 800 Ci/mmol; 1 Ci = 37 GBq; Amersham). Cells were harvested in phosphate-buffered saline and lysed using phosphate-buffered saline containing 0.5% (wt/vol) sodium deoxycholate and 0.5% (vol/vol) Nonidet P-40; postnuclear supernatants were recovered after centrifugation in a Microfuge.

Immunoprecipitation and Glycosidase Treatment. For immunoprecipitation, we used a rabbit antiserum raised against hCBG that had been purified from human serum. (Both the antiserum and purified hCBG were kind gifts of R. Kuhn of Hana Biologics.) Five microliters of this antiserum quantitatively immunoprecipitates hCBG from 2×10^6 infected Sf9 cells. Immunoprecipitation was carried out as described (17). To remove asparagine-linked carbohydrates, immunoreactive products were digested with endoglycosidase H or N-Glycanase according to manufacturer's protocols (Genzyme). Radiolabeled proteins were resolved in 12.5% acrylamide/SDS gels, which were then subjected to fluorography and exposed to Kodak XAR film at −70°C. Quantitation was by densitometric scanning of the fluorograms.

[^{3}H]Cortisol Binding Assay. Scatchard analysis and saturation binding measurements were made using [^{3}H]cortisol (40–50 Ci/mmol; New England Nuclear) in the filter assay described by Schiller and Petra (18). Equilibrium association constants were determined by competition with unlabeled steroids (19). Sf9 cell lysates, human serum, and purified hCBG derived from human serum were assayed directly after dilution in 10 mM Tris·HCl at pH 7.4. Media samples were concentrated and dialyzed against the same buffer.

RESULTS

***In Vitro* Synthesis and Modification of Recombinant hCBG.** A full-length hCBG cDNA was isolated from a human liver cDNA library, using hybridization probes derived from the hCBG cDNA sequence reported by Hammond *et al.* (3). mRNA transcribed *in vitro* from this cDNA was used to program protein synthesis in the reticulocyte lysate-derived system, and the [^{35}S]methionine-labeled products were subjected to immunoprecipitation with anti-hCBG antibodies. The primary translation product is an immunoreactive 42-kDa polypeptide (S^+; Fig. 1*A*, lane 1). Addition of canine pancreatic microsomes causes the appearance of several higher molecular mass forms of 43–50 kDa (GP; Fig. 1*A*, lane 2). These forms are not digested by exogenously added protease (Fig. 1*A*, lanes 3 and 4), indicating that they are localized within the lumen of the microsomal vesicles. Removal of asparagine-linked carbohydrates by treatment with endoglycosidase H converts these larger forms to a 39-kDa species, indicating cleavage of the amino-terminal signal peptide (S^-; Fig. 1*A*, lane 5). The *in vitro* protein product of this cDNA thus displays the molecular mass, immunoreac-

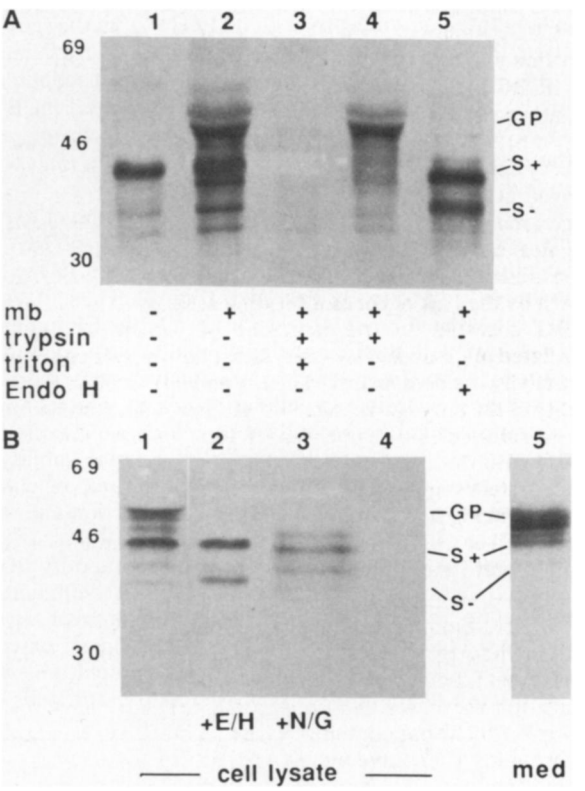

FIG. 1. Expression of recombinant hCBG *in vitro* and *in vivo*. [^{35}S]Methionine-labeled hCBG derived from cell-free translation (*A*) or Sf9 cells (*B*) was immunoprecipitated with anti-hCBG antiserum and resolved on SDS/12% PAGE. (*A*) *In vitro*-transcribed hCBG mRNA was translated in a reticulocyte lysate-derived system in the absence (lane 1) or presence (lanes 2–5) of canine pancreatic microsomes (mb). After translation, the reactions were incubated with trypsin (100 μg/ml, 30 min, 0°C) in the presence (lane 3) or absence (lane 4) of 0.5% (vol/vol) Triton X-100. Lane 5, the immunoprecipitate was incubated with endoglycosidase H (Endo H) prior to electrophoresis. (*B*) Sf9 cells infected with a baculovirus encoding hCBG (lanes 1–3 and 5) or a wild-type baculovirus (lane 4) were metabolically labeled with [^{35}S]methionine for 2 h at 66 h postinfection, and cell lysates (lanes 1–4) or medium (med; lane 5) were subjected to immunoprecipitation. The immunoprecipitates shown in lane 1 were treated with endoglycosidase H (+E/H; lane 2) or N-Glycanase (+N/G; lane 3) prior to electrophoresis. GP, S^+, and S^- refer to glycosylated hCBG, signal-containing pre-hCBG, and signal-cleaved unglycosylated hCBG, respectively. In *B*, the *in vitro* products were electrophoresed in an adjacent lane (not shown), and we consistently observe comigration of the respective *in vitro* and *in vivo* forms. Positions of molecular mass standards (in kDa) are indicated.

tivity, cotranslational targeting, and covalent modifications predicted for hCBG.

Synthesis and Secretion of hCBG by Insect Cells. hCBG cDNA was incorporated into a recombinant baculovirus so that its expression would be driven by the strong viral polyhedrin promoter. The recombinant virus was used to infect Sf9 cells at a multiplicity of 25 plaque-forming units per cell, and hCBG synthesis was monitored by radiolabeling and immunoprecipitation (Fig. 1*B*). During a 2-h pulse with [^{35}S]methionine, a high level of hCBG-immunoreactive material was synthesized, corresponding to 2–5% of the total cellular protein synthesis (Fig. 1*B*, lane 1). All of the molecular forms identified by *in vitro* translation are detected in infected cell lysates. Typically, we observe 50–75% of the total hCBG immunoreactivity in the higher molecular mass glycosylated forms of hCBG (GP), which are substrates for endoglycosidase H and N-Glycanase (Fig. 1*B*, lanes 2 and 3). A small amount of the signal peptide-cleaved form of hCBG (S^-) is synthesized, and the remainder is represented in the signal peptide-containing form of hCBG (S^+). In the secreted fraction, the glycosylated forms predominate (Fig. 1*B*, lane 5; Fig. 4*A*, lane 3), and these polypeptides are susceptible to endoglycosidase H digestion (data not shown). GP-hCBG is secreted with a $t_{1/2}$ of 90 min; at the end of a 5-h chase, ≈60% of the total GP-hCBG is extracellular, and this material is relatively stable (Fig. 2).

The intracellular level of S^+-hCBG varies among experiments, and its behavior is distinct from that of GP-hCBG. The pulse-labeled S^+-hCBG species rapidly appears in the medium and is subsequently degraded (Fig. 2). These data are consistent with inefficient translocation into the ER lumen or, alternatively, with inefficient signal peptide cleavage following translocation. In either case, the unglycosylated precursor could be susceptible to cellular proteases, and its apparent secretion might be secondary to cell damage caused by viral infection. For our purposes, the relative lability of S^+-hCBG after externalization supports the use of culture medium as a source of GP-hCBG for purification and analysis.

A competitive immunoassay was used to quantify hCBG production. The displacement curve of Sf9-derived material is parallel to that of hCBG purified from human serum, indicating a high degree of similarity in antigenic determinants (Fig. 3). To measure secretion, infected cultures were fed with fresh medium at 48 h postinfection, and cells and

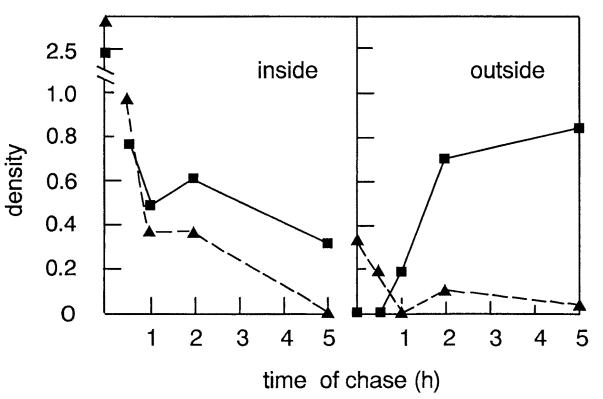

FIG. 2. Kinetics of secretion of recombinant hCBG from Sf9 cells. Sf9 cells infected with a recombinant baculovirus encoding hCBG were pulse-labeled for 1 h with [^{35}S]methionine at 100 μCi/ml and chased in the presence of 10 mM unlabeled methionine. hCBG was immunoprecipitated from cell lysates and media, resolved on SDS/PAGE, and quantified by densitometry. The values shown for intracellular (inside) and medium (outside) hCBG are derived from equal numbers of cells, allowing them to be directly compared. ■, GP-hCBG; ▲, S^+-hCBG.

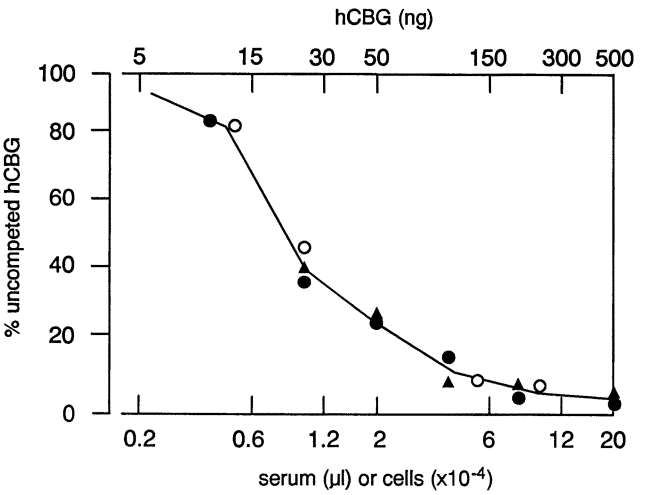

FIG. 3. Immunoquantitation of hCBG production. [^{35}S]Methionine-labeled translation products (1 × 10^5 cpm; as in Fig. 1, lane 1) were incubated with sufficient polyclonal anti-hCBG antiserum to achieve 50% immunoprecipitation, in the presence of the indicated amounts of purified human serum hCBG (●), human serum (▲), and Sf9 cell lysate (○). The immunoprecipitates were resolved on SDS/PAGE, and the amount of radiolabeled hCBG recovered in antigen–antibody complexes was quantified by densitometry.

media were harvested at 90 h. Under these conditions, 10^6 infected cells contain 2.3–3 μg (58–75 pmol) of immunoreactive hCBG, and media derived from 10^6 cells contain 1 μg (25 pmol) of hCBG (Table 1); this corresponds to the secretion of ≈1–2 μg per ml per 24 h in monolayer culture.

Biological Activity of Recombinant hCBG. The steroid-binding properties of hCBG derived from Sf9 cells and human serum were compared by using saturation binding and Scatchard analyses (18, 19). The determination of active hCBG in human serum by saturation binding of [^{3}H]cortisol correlates well with the amount measured by immunoassay (Table 1). While uninfected Sf9 cells have negligible [^{3}H]cortisol-binding activity (data not shown), infected cultures contain 10–25 pmol per 10^6 cells. The secreted fraction contains 75–90% of the total activity, and this material binds 0.7–0.8 mol of cortisol per mol of immunoreactive hCBG (Table 1). Most importantly, the affinity of the secreted recombinant protein for cortisol is indistinguishable from that of serum hCBG, as is the rank order of binding of other steroids (Table 2). These data indicate that Sf9 cells secrete authentic hCBG that contains a native steroid-binding site.

We also tested the steroid-binding capability of *in vitro*-synthesized hCBGs. Reticulocyte lysates programmed with hCBG mRNA contain 2–5 ng of hCBG per 100-μl reaction mixture; if fully active, this material would bind 40–100 fmol of [^{3}H]cortisol, which is readily detectable in our assay (see legend to Table 1). Neither the primary *in vitro* translation product (Fig. 1*A*, S^+) nor the mixture of the S^+-hCBG and

Table 1. Production of recombinant hCBG

Parameter	Human serum	Sf9 lysate	Sf9 medium
IR hCBG, pmol	50	113	60
[^{3}H]Cortisol, pmol	54	6	48
RBA	1.08	0.05	0.80

Values are for 100 μl of human pregnancy serum and for lysate and medium fractions derived from 2.5 × 10^6 Sf9 cells. Immunoreactive (IR) hCBG was quantified as described in the legend to Fig. 3. Saturation binding of [^{3}H]cortisol was measured as described in *Methods*. In 50-μl reaction mixtures, binding is linear between 25 and 250 fmol of hCBG, and the standard error is ±16%. The relative binding activity (RBA) is the ratio of [^{3}H]cortisol to immunoreactive hCBG.

Table 2. Comparison of steroid binding by recombinant and serum hCBG

	Human serum		Sf9 cells	
Steroid	K_a, M^{-1}	K_a / K_a(Cs)	K_a, M^{-1}	K_a / K_a(Cs)
Cortisol	2.6×10^8	1.0	1.8×10^8	1.0
Progesterone	4.3×10^7	0.17	2.2×10^7	0.12
Testosterone	1.8×10^6	0.007	1.0×10^6	0.006
Dexamethasone	5.5×10^4	0.0002	2.6×10^4	0.0001
Ecdysone	$<1.4 \times 10^4$	—	$<1.4 \times 10^4$	—

The association constants for cortisol binding to hCBG in human pregnancy serum and Sf9 cell medium were determined by Scatchard analysis (19, 20). Association constants for the other steroids were determined by their competition with [^{3}H]cortisol at 50% saturation of binding. The error in the association constants is a factor of 2. K_a(Cs), K_a of cortisol.

GP-hCBG forms generated by inclusion of microsomes (Fig. 1*A*, lane 2) exhibited detectable cortisol-binding activity.

Effect of Glycosylation on Steroid-Binding Activity. To examine the role of glycosylation in the production of active hCBG, infected Sf9 cells were treated with tunicamycin, which inhibits asparagine-linked glycosylation. Metabolic labeling revealed the synthesis of both S^+- and S^--hCBG (Fig. 4*A*, lane 2). As previously observed in hepatoma cells (20), unglycosylated hCBG is secreted at reduced levels relative to GP-hCBG (Fig. 4*A*, lanes 3 and 4). Longer autoradiographic exposures also indicated residual synthesis of GP-hCBG in tunicamycin-treated cells, and this was confirmed by immunoblot analysis of unlabeled medium from parallel cultures (Fig. 4*B*, lane 2). To separate S^+- and S^--hCBG from the glycosylated forms, this material was adsorbed to concanavalin A-Sepharose (Fig. 4*B*, lane 3). Saturation binding of [^{3}H]cortisol to secreted hCBG was reduced to 24% of the control value by tunicamycin treatment, and concanavalin A-purified unglycosylated hCBG was completely inactive (Fig. 4*B*).

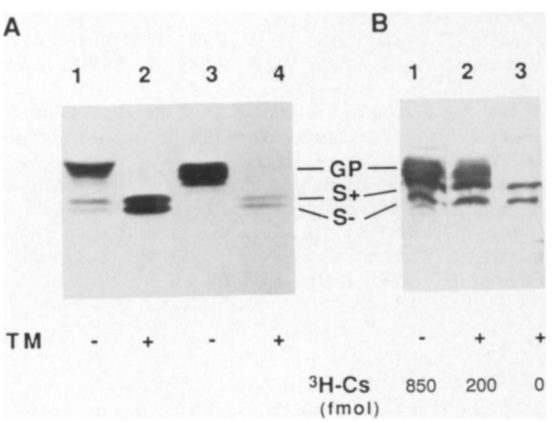

FIG. 4. Role of glycosylation in secretion of biologically active hCBG. Infected Sf9 cells were treated with or without tunicamycin (5 μg/ml) for 5 h at 40 h postinfection. In parallel cultures, media were then replaced with either methionine-free medium containing [^{35}S]methionine at 100 μCi/ml (*A*) or complete medium (*B*), lacking or containing tunicamycin (TM). In both cases, incubation was continued for an additional 16 h. (*A*) Labeled cells (lanes 1 and 2) and media (lanes 3 and 4) were harvested and subjected to immunoprecipitation with anti-hCBG antibodies, and the immunoprecipitates were resolved by SDS/PAGE. (*B*) Unlabeled culture media were harvested, concentrated, and incubated with concanavalin A-Sepharose to adsorb glycosylated polypeptides as described (21). The starting material (lanes 1 and 2) and the material that did not bind to concanavalin A (lane 3) were resolved by SDS/PAGE and identified by immunoblotting (22). Saturation binding of [^{3}H]cortisol, measured in the same amount of material as was loaded on the gel, is indicated below the corresponding lanes (^{3}H-Cs).

DISCUSSION

We report the production of high levels of biologically active hCBG in a heterologous system. Sf9 cells infected with an hCBG recombinant baculovirus secrete large quantities of glycosylated hCBG (16–24 pmol per 10^6 cells per 40 h); this level is similar to that observed in other baculovirus-directed expression systems (23). The secreted protein is functionally equivalent to human serum hCBG in its relative steroid-binding activity, high affinity for cortisol, and rank order of binding of other steroids. Large-scale production of wild-type and mutant hCBG for physical-chemical analysis and structure–function studies is therefore feasible using the baculovirus system.

Comparison of the steroid-binding properties of different molecular forms of hCBG provides a preliminary picture of how this protein acquires its native structure during biogenesis. The primary *in vitro* translation product does not bind cortisol, suggesting that signal peptide cleavage and/or core glycosylation are required for biological activity. A specific requirement for glycosylation is indicated by the observation that unglycosylated hCBG synthesized in the presence of tunicamycin, which consists of a mixture of signal-containing and signal-cleaved forms, does not bind cortisol. Subsequent addition of terminal sugars, however, which normally accompanies Golgi transport of hCBG in hepatocytes, is clearly not required for function, as shown by the endoglycosidase H sensitivity of the fully active, secreted glycoprotein. In previous studies, treatment of serum-derived native hCBG with a mixture of glycosidases, which effectively removed >90% of the bound sugar residues, did not significantly reduce cortisol binding (24). Our data support the conclusion that the complex carbohydrate moieties of hCBG are not directly involved in steroid binding. Instead, we have identified glycosylation as a critical cotranslational event that most likely influences the subsequent folding pathway(s) of hCBG within the ER lumen.

Several observations further suggest that hCBG does not achieve its final tertiary structure immediately upon arrival in the ER. *In vitro*, hCBG synthesized in the presence of microsomes does not bind cortisol, despite having undergone membrane translocation and early covalent modifications. A more striking observation is that in Sf9 cells the relative cortisol-binding activity of cell-associated hCBG is much lower than that of secreted hCBG, even after taking into account the presence of inactive unglycosylated polypeptides. That is, hCBG polypeptides in a typical Sf9 cell lysate preparation are 50–75% glycosylated (Fig. 1*B*, lane 1; Fig. 4*A*, lane 1) but exhibit only 7–10% of the native cortisol-binding capacity per mole of immunoreactive hCBG (Table 1). In contrast, the extracellular material is >90% glycosylated (Fig. 1*B*, lane 5; Fig. 4*A*, lane 3) and binds cortisol 70–80% as efficiently as serum-derived hCBG. A trivial explanation is that the cell lysate contains immunoreactive degradation products, not present in the extracellular medium, which have lost cortisol-binding activity. This seems unlikely, however, since there is a good correlation between hCBG measured by immunoassay and intact hCBG polypeptides detected by SDS/PAGE. We hypothesize that newly synthesized glycosylated molecules acquire full steroid-binding capability posttranslationally, and, once they have achieved a native structure, they are rapidly secreted. The high yield of active hCBG in the extracellular medium can then be explained by time-dependent conversion of inactive intracellular polypeptides to active extracellular ones.

The nature of this maturation process remains to be investigated. Attention has focused on the ER lumen as the

site of folding and assembly, and genetic and biochemical evidence has implicated accessory proteins, termed chaperonins, in folding and proofreading phenomena (25). Many of the documented examples involve oligomerization of integral membrane proteins and assembly of multimeric secretory complexes (25); in the case of hCBG, an analogous intramolecular folding process may occur. We suggest that formation or stabilization of a native steroid-binding site could require interaction with chaperonins. There are several candidates for such proteins in Sf9 cells (26). In this regard, an analogy may be made between hCBG and the intracellular glucocorticoid receptor (GR), which binds to a cytosolic heat shock protein, hsp90, soon after synthesis; recent evidence suggests that this association occurs via the GR steroid-binding domain (27, 28). Although GR and hCBG do not have identical steroid specificities (29), suggesting differences in their steroid-binding sites, the two proteins could share a common requirement to sequester and stabilize an unoccupied steroid-binding pocket after synthesis. In the case of hCBG, we speculate that interaction with ER components, particularly in a heterologous cell, masks its cortisol-binding activity and that its secretion is preceded by release from such a protein complex.

A related issue is whether hCBG binds cortisol intracellularly; if so, cortisol binding might be required for secretion. The solubility properties of cortisol suggest that it is freely accessible to the lumenal spaces of the ER and Golgi; thus, binding could take place in these compartments. The secretion from hepatocytes of a related protein, retinol binding protein, has been shown to require retinol binding in the ER (30), and our preliminary results in the Sf9 system suggest that the presence of cortisol increases the secretion and/or stability of hCBG (unpublished data). Since Sf9 cells do not require cortisol for growth, they provide an excellent system for investigating the possible role of ligand binding in the biogenesis of this protein.

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