Efficient eukaryotic expression system for authentic human sex hormone-binding globulin

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Sex hormone-binding globulin (SHBG) is the main carrier for androgens and oestrogens in humans. It mediates the transport of steroid hormones in the circulation and testicular fluid, and regulates their bioavailability to steroid-responsive tissues. In addition, the protein interacts with membrane receptors expressed in target tissues. Binding to the receptors is suspected to facilitate the uptake of steroid hormones and/or elicit cellular signal transduction. The identity of the SHBG receptor has not yet been resolved, in part due to a lack of sufficient quantities of authentic SHBG for receptor purification and molecular characterization. We have successfully addressed this problem by establishing an episomal expression system in human embryonic

kidney cells that produces 5 mg of fully active human SHBG per litre. The recombinant protein resembles native SHBG in terms of structure, glycosylation pattern and steroid-binding activity. Moreover, the protein interacts with plasma membranes in steroid target tissues, an activity not observed with SHBG from other recombinant expression systems. Thus our studies have removed an important obstacle to the further elucidation of the role SHBG plays in steroid hormone action.

Key words: membrane binding, protein purification, steroid carrier, steroid hormone uptake.

INTRODUCTION

In the blood and other body fluids, steroid hormones are bound in a tight complex with specific carrier proteins. One such carrier is the sex hormone-binding globulin (SHBG), a 93 kDa homodimeric protein that binds androgens and oestrogens with high affinity (reviewed in [1,2]). SHBG is produced in the liver of humans and rabbits, and in the testis of all mammalian species, where it is also known as androgen-binding protein (ABP) [1–3]. In addition, *SHBG* gene transcripts have been detected in tissues of the female reproductive tract, including the oviduct, endometrium and placenta [4,5].

It is now well appreciated that SHBG plays a pivotal role in regulating the metabolism and action of sex steroids. Foremost, the protein acts as an essential carrier for the transport of lipophilic steroids in aqueous compartments of the organism, such as blood and testicular fluid. Due to their high affinity for SHBG (K_d 0.2–4 nM), the majority of circulating androgens and oestrogens are present in the protein-bound rather than the free form *in vivo*. Steroid hormones enter cells mainly by passive diffusion through the plasma membrane, and the physiological concentration of SHBG thus plays a key role in regulating the bioavailability of its steroid ligands [6].

More recent studies have identified even more intriguing, yet poorly defined, roles for SHBG in steroid hormone action. Evidence has been presented that SHBG interacts directly with membrane receptors expressed in steroid target tissues. Binding

sites for SHBG have been detected in membrane preparations of prostate, testis, epididymis and endometrium [7–10], among others. Several hypotheses about the function of such ‘SHBG receptors’ have been advanced; for example, interaction of SHBG with cell surface receptors may sequester carrier-bound steroids on the plasma membrane, thereby enhancing diffusion of the hormone into the cells [11]. Alternatively, SHBG may be taken up directly via endocytic receptors, delivering bound steroids to the interior of the cell [12]. The latter hypothesis is supported by the demonstration of endocytic uptake of SHBG in epithelial cells of the epididymis and endometrium [13–15], and in MCF-7 breast cancer cells [16]. Finally, studies from several laboratories provide convincing evidence that binding of SHBG to membrane receptors results in an increase in intracellular cAMP levels, suggesting a direct role for the protein in signal transduction [17–20].

Despite intensive efforts, no SHBG receptor has yet been identified at the molecular level. This lack of success may be attributed in part to the technical difficulties in producing sufficient amounts of carrier protein for affinity purification of receptors and other biochemical assays. Both prokaryotic and eukaryotic expression systems for recombinant SHBG have been established [21–27]. However, some expression systems provide low yields [23–25], while lack of correct post-translational modification limits the usefulness of others [21,22,26,27]. In particular, all of the recombinant SHBG preparations reported so far exhibit major differences in glycosylation pattern as

Abbreviations used: ABP, androgen-binding protein; DHT, 5 α -dihydrotestosterone; HPS, human pregnancy serum; SHBG, sex hormone-binding globulin; rSHBG, recombinant SHBG (authentic polypeptide sequence of mature human protein preceded by the amino acid motif Ala-Pro-Leu-Ala); His-SHBG, His-tagged SHBG (SHBG sequence preceded by the osteonectin signal peptide, a hexa-histidine tag, a Myc epitope and a factor Xa cleavage site); cSHBG, cleaved SHBG (authentic SHBG polypeptide preceded by the amino acid motif Gly-Leu-Ala, produced by cleavage of His-SHBG by factor Xa); mSHBG, native SHBG purified from the plasma of mice that are transgenic for the human carrier gene.

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compared with the native protein. Because oligosaccharide side chains are considered essential for proper activity of the carrier [1,28–30], the expressed proteins may be of limited use in the study of all aspects of SHBG function.

In this study, we present a novel efficient expression and one-step purification system for SHBG using episomal expression vectors in human embryonic kidney cells. SHBG produced in this system resembles the native human protein with respect to structure, glycosylation pattern and steroid hormone binding, as well as interaction with membrane receptors.

EXPERIMENTAL

Materials

Rabbit polyclonal antibodies directed against human SHBG were purchased from Eurodiagnostica or produced by immunization with recombinant SHBG. Testosterone, 17β -oestradiol and 5α -dihydrotestosterone (DHT) were obtained from Sigma; [1,2,6,7- ^3H]testosterone and [1,2- ^3H]DHT were from Amersham/Pharmacia. Human pregnancy serum (HPS) was collected from healthy volunteers. Murine serum containing human SHBG, or SHBG purified therefrom (mSHBG), was obtained from the transgenic mouse line *shbg*11 [31,32].

Generation of SHBG expression constructs

Human SHBG cDNA was amplified by PCR from the cloned sequence (GenBank accession no. NM-001040) using primers 5'-GCCCGCTAGCCCTGAGACCTGTTCTCCCCACCCAGAG-3' and 5'-CAATACCTCGAGTTACTAATGGGAAGC-GTCAGTGCCATTGCCTGG-3', and inserted into expression vectors pCEP-Pu-SP and pCEP-Pu-SP-his-myc-fX, driving gene expression under control of the cytomegalovirus promoter [33]. In vector pCEP-Pu-SP, the endogenous signal peptide of SHBG was replaced by the corresponding sequence of the human osteonectin gene. SHBG produced in this vector [referred to as recombinant SHBG (rSHBG)] harbours the authentic polypeptide sequence of the mature human protein preceded by the amino acid motif Ala-Pro-Leu-Ala. In vector pCEP-Pu-SP-his-myc-fX, the SHBG sequence is preceded by the osteonectin signal peptide, a hexa-histidine tag, a Myc epitope and a factor Xa cleavage site (His-SHBG). Cleavage of His-SHBG with factor Xa generates the authentic SHBG polypeptide preceded by the amino acid motif Gly-Leu-Ala [referred to as cleaved SHBG (cSHBG)]. The integrity of the expression constructs was confirmed by sequencing. No adverse effects of the additional amino acids on the activity of recombinant SHBG was observed.

Expression and purification of SHBG

Human embryonic kidney cells (293-EBNA cells; Invitrogen) were electroporated with the various expression constructs and grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 250 $\mu\text{g}/\text{ml}$ G-418 sulphate (Gibco-BRL) and 1 $\mu\text{g}/\text{ml}$ puromycin (Sigma). For production of conditioned medium, the cells were plated out in 150 mm culture dishes at 80% confluency and kept at 37 °C in Dulbecco's modified Eagle's medium without fetal calf serum or Phenol Red (harvesting medium). The medium was recovered after 3 days and replaced with new harvesting medium. Typically, three to four harvests were collected before the cells detached from the culture dish.

For purification of rSHBG, 25 ml of conditioned medium was mixed with an equal volume of buffer A (20 mM Tris/HCl, 10% glycerol and 1 μM testosterone, pH 8.0) and applied to a Mono

Q HR 5/5 column (Pharmacia/Amersham) pre-equilibrated in buffer A. The column was washed with 2 vol. of buffer A. Subsequently, proteins were eluted by applying two consecutive salt gradients using buffer B (20 mM Tris/HCl, 1 M NaCl, 10% glycerol and 1 μM testosterone, pH 8.0): gradient 1 (0–25% buffer B over 50 min at a flow rate of 0.5 ml/min) and gradient 2 (25–100% buffer B over 5 min at 1 ml/min). His-SHBG was purified by standard nickel affinity chromatography according to the manufacturer's recommendations (Qiagen, Hilden, Germany). To remove the N-terminal protein tag, 40 μg of His-SHBG was incubated with 0.1 μg of factor Xa for 18 h at 24 °C in 50 mM Tris/HCl, pH 7.5, and 150 mM NaCl. N-linked carbohydrate side chains were removed by treatment with N-glycosidase F for 18 h at 37 °C in PBS containing 0.05% SDS, 0.25% Triton X-100, 5 mM dithiothreitol and 10 mM EDTA.

PAGE analysis

Conventional one-dimensional SDS/PAGE and subsequent staining of protein bands with silver nitrate or Coomassie Brilliant Blue G (Sigma) was performed according to standard protocols. Enhanced chemiluminescence (Amersham/Pharmacia) was used for Western blot analysis. For characterization of native proteins, the samples were subjected to non-denaturing PAGE (4% stacking and 12% resolving gels) containing 100 μM DHT as published previously [21]. For two-dimensional PAGE, horizontal first-dimensional isoelectric focusing was performed using immobilized pH gradients strips (Amersham/Pharmacia). Approx. 200 μg of protein was loaded on to the immobilized non-linear pH 3 to pH 10 gradient strips using a re-swell tray. Vertical second-dimension gels (5% stacking and 12% resolving) were run as described [34].

Steroid-binding assay

Binding of steroid hormones to serum samples or purified carrier proteins was carried out as published [35]. Briefly, 2 pmol of ^3H -labelled steroid was incubated in a 300 μl sample volume with 800 fmol of protein, preincubated for 30 min at room temperature with dextran-coated charcoal to remove any prebound steroids. Where indicated, the reaction buffer also included unlabelled steroids as competitors. The sample mixture was incubated for 60 min at room temperature. Thereafter, free steroids were extracted by treatment with dextran-coated charcoal for 10 min at 4 °C followed by centrifugation (3000 g for 10 min), and the amount of protein-bound radioactivity was determined.

CD spectra

Protein samples were dialysed against 10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 1 μM ZnCl_2 and 1 μM testosterone (buffer 1), and protein concentrations were determined thereafter. CD spectra were measured using a Jasco J-720 spectropolarimeter at 25 °C. Data were recorded every 0.5 nm using a 4 s averaging time and a 1.0 nm bandwidth. Spectra were averaged three times, and the buffer baseline measured under the same conditions was subtracted. The sample concentration was 0.7 mg/ml in buffer 1, and the path length of the sample cell was 0.0094 cm. Mean molar residue ellipticities (θ ; degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$) were calculated using mean residue masses of 108.3 Da for rSHBG, cSHBG and SHBG purified from transgenic mouse serum, and 108.4 Da for His-SHBG. The content of secondary structure was calculated from far-UV spectra using the program CONTIN [36].

SHBG membrane-binding assay

Purified recombinant SHBG was labelled with ^{125}I using the Iodo-Gen[®] method [specific radioactivity $(13\text{--}15) \times 10^3$ c.p.m./ng] [37]. Binding of ^{125}I -SHBG to purified membranes from bovine epididymis and endometrium was performed essentially as described [38]. Briefly, 400 pg of ^{125}I -SHBG was mixed with 600 μg of purified membranes in a total volume of 200 μl of reaction buffer [10 mM Hepes, pH 7.4, 2 mM CaCl_2 , 1 mM MgCl_2 , 1% (w/v) BSA, 1 μM ZnCl_2 , 1 μM testosterone and 30 $\mu\text{g}/\text{ml}$ digitonin]. In addition, the reaction mixture included the indicated concentrations of unlabelled SHBG. The reaction mixture was incubated at 4 °C for 18 h and then precipitated on Millipore filters (GVWP 293 2A) using a vacuum suction device. The filters were washed extensively with reaction buffer and the amount of bound radioactivity was determined.

RESULTS AND DISCUSSION

A number of prokaryotic and eukaryotic SHBG expression systems have been developed previously [21–27]. Expression in *Escherichia coli* provided an inexpensive and efficient system for producing various domains of SHBG. With the help of bacterially expressed SHBG fragments, the residues involved in steroid hormone binding and dimerization were mapped [21,22]. A bacterial expression system was also used to produce the N-terminal laminin G-like domain of the protein, and to solve its crystal structure [39]. While expression in *E. coli* worked well for fragments of SHBG, the unmodified 45 kDa full-length protein could only be produced in low yield because of excessive premature translation termination [21]. Another drawback of the bacterial system is the lack of glycosylation, since this is considered essential for the full biological activity of the carrier [1,28–30].

For the reasons mentioned above, we decided to express full-length human SHBG in a eukaryotic expression system, and focused our attention on expression vectors based on the Epstein–Barr virus genome. Two genetic elements, the viral origin of replication and the gene for nuclear antigen EBNA-1, are sufficient to sustain high-copy-number episomal replication of recombinant vectors in primate cells [40]. Episomal propagation circumvents the need to generate cell lines stably transfected with SHBG expression constructs. Here we used pCEP-Pu vectors [33] to generate two constructs for the production of SHBG (Figure 1). To facilitate purification of the recombinant protein by affinity chromatography, the human SHBG cDNA

Figure 1 Structure of SHBG expression constructs

The structural organization of SHBG expression constructs is shown. Human SHBG was expressed as a fusion protein carrying a hexa-histidine tag (His6), a Myc epitope and a factor Xa cleavage site (His-SHBG; upper construct). cSHBG was produced by removal of the N-terminal tags from His-SHBG by factor Xa treatment. Alternatively, SHBG was expressed as untagged recombinant protein (rSHBG; lower construct). Details of the cloning procedure are given in the Experimental section.

Figure 2 Purification of rSHBG from 293 cells

(A) Conditioned medium from rSHBG-expressing 293 cells was subjected to anion-exchange chromatography. Fractions eluting from the column were tested for absorbance at 280 nm (OD_{280} ; solid line) and for binding of ^3H testosterone (dashed peak). The stepped dashed line indicates the salt gradient (0–1 M NaCl) used for protein elution. (B) Selected fractions were separated by reducing SDS/PAGE (4–12% gels) and stained with silver nitrate. As a reference, an aliquot of the conditioned medium was tested in parallel (S). The positions of marker proteins in the gel are indicated in kDa. The arrow points to the major protein band corresponding to rSHBG.

was expressed as a fusion protein containing a hexa-histidine tag, a Myc epitope and a factor Xa cleavage site, followed by the mature SHBG polypeptide sequence. This protein is referred to as His-SHBG. The N-terminal modifications can be removed from the fusion protein by treatment with factor Xa, generating cSHBG. Alternatively, we expressed human SHBG as an untagged polypeptide (Figure 1). This protein is referred to as rSHBG.

To express SHBG, we transfected human embryonic kidney cells (293 cells) with the expression constructs and recovered conditioned culture medium containing secreted SHBG. The cells were cultured in a medium without serum or other supplements, to minimize the amount of contaminating proteins. rSHBG was detectable as the major protein band in the conditioned medium (Figure 2B, fraction S). For purification of rSHBG, we developed a one-step purification protocol using anion-exchange chromatography (Figure 2A). Proteins were eluted from the column by a salt gradient and assayed for testosterone binding. rSHBG was recovered in an isolated peak around fractions 14–18 that co-eluted with the steroid-binding activity. Other proteins in the medium eluted in later fractions devoid of steroid-binding activity (Figure 2A, fractions 22–34).

Figure 3 Comparative analysis of SHBG variants

Highly purified preparations of His-SHBG (lanes 1 and 5), cSHBG (lane 2 and 6), rSHBG (lanes 3 and 7) and mSHBG (lanes 4 and 8) were subjected to reducing SDS/PAGE (4–12% gels) and staining with silver nitrate (lanes 1–4) or Western blot analysis using anti-(human SHBG) antiserum (lanes 5–8). The positions of marker proteins in the gel are indicated in kDa. The arrowhead denotes the position of the cSHBG proteolytic product generated by factor Xa cleavage of His-SHBG (lanes 2 and 6).

Therefore rSHBG-containing fractions were free of any contaminating proteins (Figure 2B, fractions 15 and 16). His-SHBG was also purified to homogeneity by a one-step purification protocol using standard nickel affinity chromatography (results not shown). Approx. 5 mg of His-SHBG or rSHBG was recovered from 1 litre of culture medium of cells grown either in standard 150 mm culture dishes or in a continuous fermenter setting (results not shown). The quality of the SHBG preparations was evaluated by SDS/PAGE followed by staining with silver nitrate or Western blot analysis (Figure 3). Preparations of His-SHBG (Figure 3, lane 1) and rSHBG (lane 3) were absolutely pure. Removal of the N-terminal tag from His-SHBG (to produce cSHBG) was achieved by treatment of the protein with factor Xa (Figure 3, lane 2). A minor degradation product was visible in the cSHBG preparation. This protein fragment was derived from His-SHBG by cryptic protease digestion, as shown by Western blot analysis (Figure 3, lanes 5–8). Interestingly, cSHBG and rSHBG exhibited the same electrophoretic mobility in the gel as native SHBG purified from the plasma of mice that are transgenic for the human carrier gene (mSHBG) [31]. This finding is noteworthy, because previously reported preparations of recombinant SHBG were characterized by an altered electrophoretic mobility, indicating changes in glycosylation pattern as compared with the native carrier protein [26,27].

So far, our studies had identified an efficient expression and purification system for the production of large amounts of full-length human SHBG. The expression of SHBG in established mammalian cell lines has been reported before [23–25]. However, production in Chinese hamster ovary or BHK-21 cells was inefficient and did not allow purification of significant amounts of the protein. In contrast with mammalian cells, efficient expression systems for SHBG have been developed in insect cells [26] and in the yeast *Pichia pastoris* [27]. The levels of protein production were similar to those observed for 293 cells (1–4 mg/l). However, the protein expressed in insect or yeast cells was characterized by an abnormal glycosylation pattern, limiting its use for studying SHBG activity.

To confirm the authenticity of our SHBG, we studied the glycosylation pattern of the protein. The identical molecular masses of recombinant and native SHBG preparations had already indicated an authentic glycosylation status of the expressed proteins (Figure 3). Furthermore, the His-SHBG and rSHBG preparations were composed of two main protein bands, a major higher-molecular-mass band and a minor, smaller band

Figure 4 Analysis of the glycosylation pattern of rSHBG

Purified rSHBG was incubated in buffer with (+; lane 2) or without (–; lane 1) N-glycosidase F and subjected to reducing SDS/PAGE (4–12% polyacrylamide) and Western blot analysis. The arrowhead highlights underglycosylated SHBG present as a minor protein band in the rSHBG preparation (lane 1).

Figure 5 Analysis of SHBG variants by two-dimensional gel electrophoresis

Purified preparations of rSHBG and His-SHBG were subjected to reducing two-dimensional SDS/PAGE and Western blot analysis using anti-SHBG antiserum. As references, HPS and serum from SHBG-transgenic mice (MS) were tested in parallel.

(Figure 3, lanes 1 and 3). This feature is known as the microheterogeneity of SHBG [28], and is seen in all natural SHBG preparations (Figure 3, lane 4). Microheterogeneity reflects the existence of SHBG glycosylation variants that are believed to fulfil different functions in steroid hormone metabolism [28,29]. The lower-molecular-mass band in the rSHBG preparation probably represents the previously described underglycosylated form of the protein, as was demonstrated by treatment of rSHBG with N-glycosidase F (Figure 4). Removal of the N-linked oligosaccharide side chains resulted in a faster mobility of the high-molecular-mass band, which became identical with that of the lower band. We also compared the glycosylation variants in His-SHBG and rSHBG with those of the native protein in HPS or in serum from mice transgenic for the human protein. To do this, we analysed HPS and mouse serum samples by two-dimensional gel electrophoresis and immunodetection with anti-SHBG antibodies. In both samples, native SHBG was present as a cluster of six to eight variants, distinguished by molecular mass and pI value (Figure 5). A similar pattern of SHBG isoforms was detected when purified preparations of His-SHBG or rSHBG were analysed (Figure 5), suggesting correct glycosylation of the recombinant proteins.

Next we performed CD spectroscopy on His-SHBG, cSHBG, rSHBG and mSHBG, in order to compare the overall structural

Figure 6 CD spectroscopy of SHBG variants

His-SHBG, cSHBG, rSHBG and mSHBG were examined by CD spectroscopy, as detailed in the Experimental section.

Figure 7 Binding of $[^3\text{H}]$ testosterone to purified SHBG

Fixed amounts (800 fmol) of His-SHBG ($\square$), cSHBG ($\blacksquare$) or rSHBG ($\circ$) were incubated with 2 pmol of $[^3\text{H}]$ testosterone and the indicated concentrations of the unlabelled steroid. The amount of $[^3\text{H}]$ testosterone bound to SHBG was measured as described in the Experimental section. As a control, steroid binding to HPS was analysed in parallel ($\bullet$). All data points were determined in duplicate. Inset: samples of 5 μg of purified cSHBG (lane 1), His-SHBG (lane 2) and purified mSHBG (lane 3) were subjected to native PAGE and staining with Coomassie Blue. The bracket marks high-molecular-mass aggregates present in the His-SHBG preparation.

similarity of the various SHBG species. As seen in Figure 6, the CD spectra of the various proteins were almost identical, indicating a similar overall fold. The spectral characteristics resembled those of proteins composed mainly of β -sheets and β -turns, with a low content of α -helical structure. A content of 45%, 43% and 42% β -sheet structure was calculated for cSHBG, rSHBG and His-SHBG respectively. These data agree perfectly with the values of 43% β -strand for mSHBG (Figure 6) and 41% β -strand that was found in the three-dimensional structure of the laminin G-like domain of SHBG [39].

Having confirmed the overall structural integrity and the authentic glycosylation pattern of rSHBG, we finally tested for the two known activities of the carrier protein: the binding of steroid hormones and the interaction with membrane receptors.

Figure 8 Binding of testosterone, 17β -oestradiol and cortisol (hydrocortisone) to purified rSHBG

Fixed amounts (800 fmol) of rSHBG were incubated with 2 pmol of $[^3\text{H}]$ testosterone and the indicated concentrations of unlabelled testosterone ($\circ$), 17β -oestradiol ($\bullet$) or cortisol ($\square$). The amount of $[^3\text{H}]$ testosterone bound to the protein was determined in duplicate, and is given as a percentage of the radioactivity bound in the absence of competitor.

When saturable binding of $[^3\text{H}]$ testosterone to the proteins was assayed, no differences in activity were observed for cSHBG, rSHBG and SHBG in HPS (Figure 7). However, the His-SHBG fusion protein demonstrated a lower steroid-binding capacity. This observation was explained by the presence of high-molecular-mass aggregates in this protein preparation (Figure 7, inset). Aggregation occurred via the hexa-histidine tag of the protein, and was prevented by removal of the modification with factor Xa (Figure 7, inset). In agreement with its function as a carrier for testosterone and 17β -oestradiol, the binding of $[^3\text{H}]$ testosterone to rSHBG could be competed by testosterone and 17β -oestradiol, but not by other steroids such as cortisol (Figure 8). Scatchard analysis demonstrated very similar K_d values for DHT binding to cSHBG and native SHBG in HPS (Figure 9).

In addition to simply transporting steroid hormones in the circulation and other body fluids, SHBG may also be more directly involved in modulating steroid hormone action. In particular, SHBG is suspected to interact with specific receptors

Figure 9 Scatchard analysis of DHT binding to SHBG

Scatchard plots for $[^3\text{H}]$ DHT binding to cSHBG and HPS were obtained as described in the Experimental section.

Figure 10 Saturable binding of rSHBG to membrane preparations from epididymis and endometrium

¹²⁵I-rSHBG (400 pg) was incubated with 600 μg of membrane preparations from bovine epididymis (A) or endometrium (B) in the presence of the indicated concentrations of unlabelled rSHBG. Values are given as a percentage of ¹²⁵I-rSHBG bound in the absence of competitor (100%). The data represent the means of four individual experiments (±S.E.M.). Where no error bar is shown, the values are smaller than the actual symbol.

expressed in steroid target tissues. Binding to the receptors may result in the endocytic uptake of the carrier and in the specific delivery of bound steroids to the cytoplasm of the cells. This hypothesis is supported by the fact that endocytic uptake of SHBG has been demonstrated in steroid-responsive tissues, such as epididymis and endometrium [13–15]. Furthermore, the existence of endocytic pathways for the cellular uptake of other steroid-carrier complexes has been described previously [41]. A biological system in which the endocytic uptake of SHBG may play an important physiological role is the delivery of testosterone to the principal cells in the epididymis. In the male genital tract, SHBG (ABP) is produced by Sertoli cells in the testis and secreted into the lumen of the seminiferous tubules [13,42]. There it binds testosterone, present in large amounts in the testicular fluids. Via the efferent ducts, SHBG is transported to the epididymis and internalized by principal cells lining the epididymal duct [13,14]. Because the principal cells in the epididymis are responsible for the conversion of testosterone into DHT that is required for sperm maturation, endocytic uptake of SHBG may be a route to deliver testosterone to these cells [42,43]. Unfortunately, the identity of the cell surface SHBG receptor has not yet been uncovered. Binding studies indicate that the receptor binds SHBG with a K_d of 50 nM when assayed in membrane suspensions, and a K_d of 0.5 μM in solubilized membranes [10]. By size chromatography, an approximate molecular mass of 174 kDa was calculated [10].

In previous studies, native SHBG purified from HPS was used to screen for membrane receptors [7–10]. To test whether our rSHBG interacted with SHBG receptors, we assayed the binding of rSHBG to bovine epididymis and endometrium membranes permeabilized in digitonin. Binding was performed in the presence of testosterone, to achieve steroid-carrier complex-formation. As seen in Figure 10, we observed saturable binding of ¹²⁵I-labelled rSHBG to both membrane preparations. The affinity of binding to the permeabilized epididymis membranes was in

perfect agreement with published data for the native protein (K_d 0.5 μM). At present, we can only speculate on the physiological significance of this SHBG-binding site in the epididymis. Because it binds SHBG in the presence of testosterone, it may be involved in facilitating the uptake of steroid-carrier complexes into this tissue. For the same reason, this binding site is clearly distinct from an SHBG receptor identified in MCF-7 breast cancer cells, which binds the carrier only when it is free of steroids [17–20].

In summary, we have developed an SHBG expression and purification system that combines all the advantages of previously reported systems, but lacks their drawbacks. Expression in 293 cells constitutes an efficient, easy-to-use system for the production of large quantities of recombinant SHBG. The protein can be purified to homogeneity in a one-step purification protocol using affinity or anion-exchange chromatography. The produced protein resembles native human SHBG in terms of molecular mass, microheterogeneity, glycosylation pattern and secondary structure. Furthermore, it exhibits the same functional characteristics in terms of steroid hormone and membrane receptor binding. Thus this expression system may be superior to others for studying SHBG structure and function. It will be useful for the production of the large quantities of the carrier that will be required for identifying SHBG receptors or solving the crystal structure of the full-length protein.

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REFERENCES

- Hammond, G. L. and Bocchinfuso, W. P. (1995) Sex hormone-binding globulin/androgen-binding protein: steroid-binding and dimerization domains. *J. Steroid Biochem. Mol. Biol.* **53**, 543–552.
- Joseph, D. R. (1994) Structure, function, and regulation of androgen-binding protein/sex hormone-binding globulin. *Vitam. Horm.* **49**, 197–280.
- Reventos, J., Hammond, G. L., Crozat, A., Brooks, D. E., Gunsalus, G. L., Bardin, C. W. and Musto, N. A. (1988) Hormonal regulation of rat androgen-binding protein (ABP) messenger ribonucleic acid and homology of human testosterone-estradiol-binding globulin and ABP complementary deoxyribonucleic acids. *Mol. Endocrinol.* **2**, 125–132.
- Misao, R., Itoh, N., Mori, H., Fujimoto, J. and Tamaya, T. (1994) Sex hormone-binding globulin mRNA levels in human uterine endometrium. *Eur. J. Endocrinol.* **131**, 623–629.
- Larrea, F., Diaz, L., Carino, C., Larriva-Sahd, J., Carrillo, L., Orozco, H. and Ulloa-Aguirre, A. (1993) Evidence that human placenta is a site of sex hormone-binding globulin gene expression. *J. Steroid Biochem. Mol. Biol.* **46**, 497–505.
- Mendel, C. M. (1989) The free hormone hypothesis: a physiologically based mathematical model. *Endocr. Rev.* **10**, 232–274.
- Krupenko, S. A., Krupenko, N. I. and Danzo, B. J. (1994) Interaction of sex hormone-binding globulin with plasma membranes from the rat epididymis and other tissues. *J. Steroid Biochem. Mol. Biol.* **51**, 115–124.
- Hryb, D. J., Khan, M. S. and Rosner, W. (1985) Testosterone-estradiol-binding globulin binds to human prostatic cell membranes. *Biochem. Biophys. Res. Commun.* **128**, 432–440.
- Porto, C. S., Abreu, L. C., Gunsalus, G. L. and Bardin, C. W. (1992) Binding of sex-hormone-binding globulin (SHBG) to testicular membranes and solubilized receptors. *Mol. Cell. Endocrinol.* **89**, 33–38.
- Frairia, R., Fortunati, N., Berta, L., Fazzari, A., Fissore, F. and Gaidano, G. (1991) Sex steroid binding protein (SBP) receptors in estrogen sensitive tissues. *J. Steroid Biochem. Mol. Biol.* **40**, 805–812.
- Avvakumov, G. V. (1991) Membrane recognition of steroid-glycoprotein complexes: a model for steroid delivery to the target cell. In *Lectins and Cancer* (Gabijs, H. J. and Gabius, S., eds.), pp. 263–272. Springer-Verlag, Heidelberg.
- Porto, C. S., Lazari, M. F., Abreu, L. C., Bardin, C. W. and Gunsalus, G. L. (1995) Receptors for androgen-binding proteins: internalization and intracellular signalling. *J. Steroid Biochem. Mol. Biol.* **53**, 561–565.

- 13 Feldman, M., Lea, O. A., Petrusz, P., Tres, L. L., Kierszenbaum, A. L. and French, F. S. (1981) Androgen-binding protein. Purification from rat epididymis, characterization, and immunocytochemical localization. *J. Biol. Chem.* **256**, 5170–5175
- 14 Gerard, A., Khanfri, J., Gueant, J. L., Fremont, S., Nicolas, J. P., Grignon, G. and Gerard, H. (1988) Electron microscope radioautographic evidence of in vivo androgen-binding protein internalization in the rat epididymis principal cells. *Endocrinology* **122**, 1297–1307
- 15 Noe, G., Cheng, Y. C., Dabike, M. and Croxatto, H. B. (1992) Tissue uptake of human sex hormone-binding globulin and its influence on ligand kinetics in the adult female rat. *Biol. Reprod.* **47**, 970–976
- 16 Porto, C. S., Gunsalus, G. L., Bardin, C. W., Phillips, D. M. and Musto, N. A. (1991) Receptor-mediated endocytosis of an extracellular steroid-binding protein (TeBG) in MCF-7 human breast cancer cells. *Endocrinology* **129**, 436–445
- 17 Ding, V. D., Moller, D. E., Feeney, W. P., Didolkar, V., Nakhla, A. M., Rhodes, L., Rosner, W. and Smith, R. G. (1998) Sex hormone-binding globulin mediates prostate androgen receptor action via a novel signaling pathway. *Endocrinology* **139**, 213–218
- 18 Nakhla, A. M., Khan, M. S. and Rosner, W. (1990) Biologically active steroids activate receptor-bound human sex hormone-binding globulin to cause LNCaP cells to accumulate adenosine 3',5'-monophosphate. *J. Clin. Endocrinol. Metab.* **71**, 398–404
- 19 Nakhla, A. M., Leonard, J., Hryb, D. J. and Rosner, W. (1999) Sex hormone-binding globulin receptor signal transduction proceeds via a G protein. *Steroids* **64**, 213–226
- 20 Fortunati, N., Raineri, M., Cignetti, A., Hammond, G. L. and Frairia, R. (1998) Control of the membrane sex hormone-binding globulin-receptor (SHBG-R) in MCF-7 cells: effect of locally produced SHBG. *Steroids* **63**, 282–284
- 21 Hildebrand, C., Bocchinfuso, W. P., Dales, D. and Hammond, G. L. (1995) Resolution of the steroid-binding and dimerization domains of human sex hormone-binding globulin by expression in *Escherichia coli*. *Biochemistry* **34**, 3231–3238
- 22 Lee, W. M., Wong, A. S., Tu, A. W., Cheung, C. H., Li, J. C. and Hammond, G. L. (1997) Rabbit sex hormone binding globulin: primary structure, tissue expression, and structure/function analyses by expression in *Escherichia coli*. *J. Endocrinol.* **153**, 373–384
- 23 Hagen, F. S., Arguelles, C., Sui, L. M., Zhang, W., Seidel, P. R., Conroy, S. C. and Petra, P. H. (1992) Mammalian expression of the human sex steroid-binding protein of plasma (SBP or SHBG) and testis (ABP). Characterization of the recombinant protein. *FEBS Lett.* **299**, 23–27
- 24 Bocchinfuso, W. P., Warmels-Rodenhiser, S. and Hammond, G. L. (1991) Expression and differential glycosylation of human sex hormone-binding globulin by mammalian cell lines. *Mol. Endocrinol.* **5**, 1723–1729
- 25 Sui, L. M., Cheung, A. W., Namkung, P. C. and Petra, P. H. (1992) Localization of the steroid-binding site of the human sex steroid-binding protein of plasma (SBP or SHBG) by site-directed mutagenesis. *FEBS Lett.* **310**, 115–118
- 26 Sui, L. M., Wong, C. and Petra, P. H. (1995) Over-expression of human sex steroid-binding protein (hSBP/hABP or hSHBG) in insect cells infected with a recombinant baculovirus. Characterization of the recombinant protein and comparison to the plasma protein. *J. Steroid Biochem. Mol. Biol.* **52**, 173–179
- 27 Sui, L. M., Lennon, J., Ma, C., McCann, I., Woo, I. and Petra, P. H. (1999) Heterologous expression of wild type and deglycosylated human sex steroid-binding protein (SBP or SHBG) in the yeast, *Pichia pastoris*. Characterization of the recombinant proteins. *J. Steroid Biochem. Mol. Biol.* **68**, 119–127
- 28 Danzo, B. J. and Bell, B. W. (1988) The microheterogeneity of androgen-binding protein in rat serum and epididymis is due to differences in glycosylation of their subunits. *J. Biol. Chem.* **263**, 2402–2408
- 29 Danzo, B. J. and Black, J. H. (1990) Structure of asparagine-linked oligosaccharides on human and rabbit testosterone-binding globulin. *Biol. Reprod.* **42**, 472–482
- 30 Cousin, P., Dechaud, H., Grenot, C., Lejeune, H., Hammond, G. L. and Pugeat, M. (1999) Influence of glycosylation on the clearance of recombinant human sex hormone-binding globulin from rabbit blood. *J. Steroid Biochem. Mol. Biol.* **70**, 115–121
- 31 Janne, M., Deol, H. K., Power, S. G., Yee, S. P. and Hammond, G. L. (1998) Human sex hormone-binding globulin gene expression in transgenic mice. *Mol. Endocrinol.* **12**, 123–136
- 32 Avvakumov, G. V., Muller, Y. A. and Hammond, G. L. (2000) Steroid-binding specificity of human sex hormone-binding globulin is influenced by occupancy of zinc-binding site. *J. Biol. Chem.* **275**, 25920–25925
- 33 Kohfeldt, E., Maurer, P., Vannahme, C. and Timpl, R. (1997) Properties of the extracellular calcium binding module of the proteoglycan testican. *FEBS Lett.* **414**, 557–561
- 34 Vorum, H., Liu, X., Madsen, P., Rasmussen, H. H. and Honore, B. (1998) Molecular cloning of a cDNA encoding human calumenin, expression in *Escherichia coli* and analysis of its calcium-binding activity. *Biochim. Biophys. Acta* **1386**, 121–131
- 35 Hammond, G. L. and Lahteenmaki, P. L. (1983) A versatile method for the determination of serum cortisol binding globulin and sex hormone binding globulin binding capacities. *Clin. Chim. Acta* **132**, 101–110
- 36 Provencher, S. W. and Glockner, J. (1981) Estimation of globular protein secondary structure from circular dichroism. *Biochemistry* **20**, 33–37
- 37 Fraker, P. J. and Speck, J. C. (1978) Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* **80**, 849–857
- 38 Jensen, P. H., Moestrup, S. K., Sottrup-Jensen, L., Petersen, C. M. and Gliemann, J. (1988) Receptors for alpha 2-macroglobulin- and pregnancy zone protein-proteinase complexes in the human placental syncytiotrophoblast. *Placenta* **9**, 463–477
- 39 Grishkovskaya, I., Avvakumov, G. V., Sklenar, G., Dales, D., Hammond, G. L. and Muller, Y. A. (2001) Crystal structure of human sex hormone-binding globulin: steroid transport by a laminin G-like domain. *EMBO J.* **19**, 504–512
- 40 Young, J. M., Cheadle, C., Foulke, Jr, J. S., Drohan, W. N. and Sarver, N. (1988) Utilization of an Epstein-Barr virus replicon as a eukaryotic expression vector. *Gene* **62**, 171–185
- 41 Nykjaer, A., Dragun, D., Walther, D., Vorum, H., Jacobsen, C., Herz, J., Melsen, F., Christensen, E. I. and Willnow, T. E. (1999) An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. *Cell* **96**, 507–515
- 42 French, F. S. and Ritzen, E. M. (1973) A high-affinity androgen-binding protein (ABP) in rat testis: evidence for secretion into efferent duct fluid and absorption by epididymis. *Endocrinology* **93**, 88–95
- 43 Turner, T. T., Miller, D. W. and Avery, E. A. (1995) Protein synthesis and secretion by the rat caput epididymidis in vivo: influence of the luminal microenvironment. *Biol. Reprod.* **52**, 1012–1019