Cytoplasmic Accumulation of Incompletely Glycosylated SHBG Enhances Androgen Action in Proximal Tubule Epithelial Cells

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Human sex hormone-binding globulin (SHBG) accumulates within the cytoplasm of epithelial cells lining the proximal convoluted tubules of mice expressing human *SHBG* transgenes. The main ligands of SHBG, testosterone and its metabolite, 5α-dihydrotestosterone (DHT), alter expression of androgen-responsive genes in the kidney. To determine how intracellular SHBG might influence androgen action, we used a mouse proximal convoluted tubule (PCT) cell line with characteristics of S1/S2 epithelial cells in which human SHBG accumulates. Western blotting revealed that SHBG extracted from PCT cells expressing a human SHBG cDNA (PCT-SHBG) is 5–8 kDa smaller than the SHBG secreted by these cells, due to incomplete N-glycosylation and absence of O-linked oligosaccharides. PCT-SHBG cells sequester [^{3}H]DHT more effectively from culture medium than parental PCT cells, and the presence of SHBG accentuates androgen-dependent activation of a luciferase reporter gene, as well as the endogenous kidney androgen-regulated protein (*Kap*) gene. After androgen withdrawal, androgen-induced Kap mRNA levels in PCT-SHBG cells are maintained for more than 2 wk *vs* 2 d in parental PCT cells. Transcriptome profiling after testosterone or DHT pretreatments, followed by 3 d of steroid withdrawal, also demonstrated that intracellular SHBG enhances androgen-dependent stimulation (*e.g. Adh7*, *Vcam1*, *Areg*, *Tnfaip2*) or repression (*e.g. Cldn2* and *Osr2*) of many other genes in PCT cells. In addition, nuclear localization of the androgen receptor is enhanced and retained longer after steroid withdrawal in PCT cells containing functional SHBG. Thus, intracellular SHBG accentuates the uptake of androgens and sustains androgens access to the androgen receptor, especially under conditions of limited androgen supply. **(*Molecular Endocrinology* 25: 269–281, 2011)**

NURSA Molecule Pages: Nuclear Receptors: AR; **Ligands:** Testosterone | Dihydrotestosterone | Bicalutamide | 17β-estradiol.

Sex hormone-binding globulin (SHBG) is the high-affinity plasma transport protein for androgens and estrogens, and it modulates the amounts of free or non-protein-bound sex steroids that can access their target tissues (1). In addition to hepatocytes, which are the primary source of plasma SHBG, the single gene (*SHBG*) encoding SHBG is expressed in several other cell types (2–4), including epithelial cells lining the renal proximal convoluted tubules (PCTs) of mice that express human *SHBG* transgenes (5). Some of the human SHBG produced by these kidney cells is most likely secreted into the renal tubule because it can be detected in the urine, but substantial amounts of immunoreactive human SHBG are retained within these cells (5). This is remarkable because the human *SHBG* transcripts in kidney cells encode the SHBG precursor polypeptide that includes the signal polypeptide necessary for secretion, and they are identical in sequence to the SHBG mRNA in hepatocytes that very actively secrete almost all the SHBG they produce, and retain very little intracellular SHBG (5). Because the mouse kidney

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Abbreviations: AR, Androgen receptor; ARE, androgen response element; ARE-luc, ARE luciferase; CHO, Chinese hamster ovary; DHT, 5α-dihydrotestosterone; E2, estradiol; EGF, epidermal growth factor; FBS, fetal bovine serum; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; PCT, proximal convoluted tubule; RT, reverse transcription; SHBG, sex hormone-binding globulin; siRNA, small interfering RNA.

is a well-known site of androgen action, we set out to characterize the SHBG that accumulates within the cytoplasm of kidney epithelial cells and determine how the presence of SHBG within these cells might modulate androgen action.

In the mouse kidney, the androgen-regulated protein (*Kap*) gene is one of the most well-studied targets of androgen action (6, 7), and its up-regulation by androgens in S1–S2 segments of the PCT requires the presence of a functional androgen receptor (AR) (8–10). In female mice, the *Kap* gene is also expressed under the influence of estrogens in the same segments (S1 and S2) of the PCT (11), whereas thyroid hormone up-regulates the *Kap* gene in the S3 cells of the PCTs of both sexes (12).

Like murine Kap mRNA, human SHBG mRNA accumulates in the S1 and S2 epithelial cells of PCTs of transgenic mice, and their levels are enhanced by androgen treatment of gonadectomized male and female mice (13). Although the Kap mRNA levels in the kidneys of *SHBG* transgenic mice are similar to those of wild-type mice, even after castration and subsequent androgen treatment (Hong, E.-J., and G.L. Hammond, unpublished data), this type of *in vivo* experiment is complicated by the fact that *Kap* is abundantly expressed in the non-androgen-responsive S3 cells under the control of thyroid hormone. To circumvent this problem, we have used an immortalized mouse PCT epithelial cell line that retains the characteristics of epithelial cells in the S1–S2 region of the PCT (14, 15), and expresses the *Kap* gene in response to androgen (16). It also appears that these cells recapitulate the way that human *SHBG* transgenes are expressed in the PCTs of the mouse kidney, because expression of a human SHBG cDNA in these cells results in significant intracellular accumulations of human SHBG.

The presence of human SHBG within cells raises the obvious question of whether it promotes the internalization and actions of sex steroids, or dampens their effects by restricting steroid access to their nuclear receptors. We have explored these questions in a series of experiments that lead us to conclude that the presence of human SHBG within specific cell types, such as PCT epithelial cells, accentuates the uptake of androgens and serves as a reservoir for androgens that can be accessed by the AR, and that this may be especially important under conditions where the supply of androgens is limited.

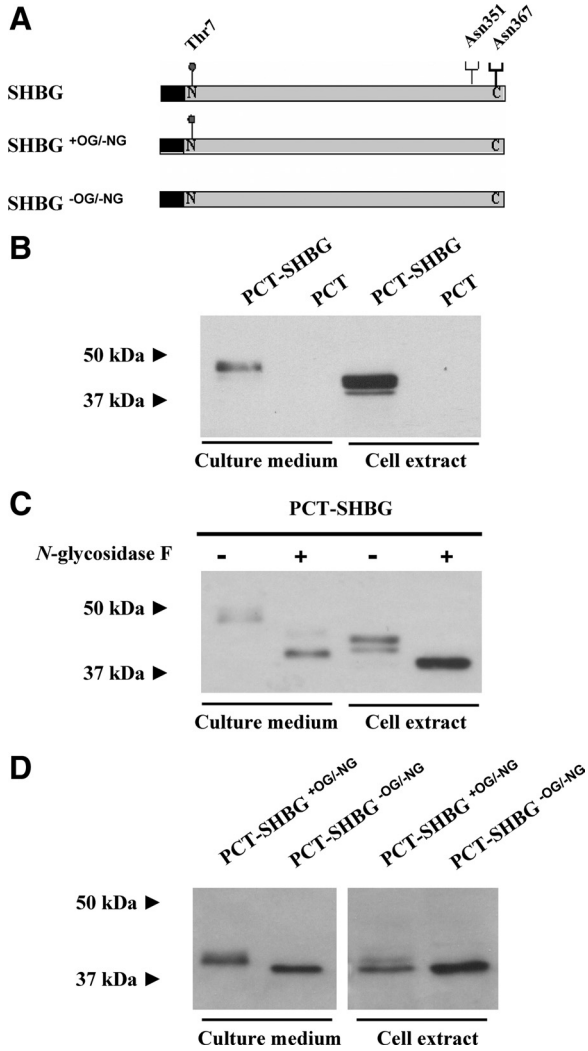

FIG. 1. Electrophoretic characteristics and glycosylation status of human SHBG in the medium and extracts of mouse PCT cells transfected with human SHBG cDNA expression vectors. A, Human SHBG contains two consensus sites for N-glycosylation (NG) at Asn^{351} and Asn^{367}, and an O-glycosylation (OG) at Thr7. B, Human SHBG in PCT-SHBG cell medium or cell extracts, detected by Western blotting, differs in terms of its electrophoretic mobility. C, A Western blot analysis of the electrophoretic microheterogeneity of SHBG produced by PCT cells in the culture medium or in the corresponding cell extracts before (−) and after (+) treatment with *N*-glycosidase F to remove N-linked oligosaccharides. D, Western blot showing differences in the apparent molecular sizes of SHBG mutants that lack NG ($SHBG^{+OG/-NG}$) or both NG and OG ($SHBG^{-OG/-NG}$) in PCT cell culture medium and cell extracts. In panels B–D, the immunoreactive wild-type SHBG or SHBG mutants in the PCT cell culture medium or cell extracts are shown on the Western blots, with the positions of protein size markers on the *left*.

Results

Human SHBG within PCT epithelial cells differs from secreted SHBG in terms of its glycosylation status

Stable transformants of PCT cells that constitutively express human SHBG or human SHBG glycosylation mutants (Fig. 1A) were used to study the biochemical properties of the human SHBG that accumulates within these cells. When examined by Western blotting, we noticed that the apparent molecular size of the SHBG extracted from PCT-SHBG cells was smaller than the SHBG in the culture medium from these cells (Fig. 1B). By comparison, and as observed previously (17), only

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trace amounts of immunoreactive SHBG were observed in cell extracts of Chinese hamster ovary (CHO) cells expressing SHBG (data not shown). The apparent sizes and electrophoretic microheterogeneity of the SHBG in PCT cell culture medium and cell extracts were quite different (Fig. 1B). We know that SHBG secreted by CHO cells is differentially glycosylated (18), and this is largely attributed to the differential utilization of the two N-glycosylation sites (at Asn351 and Asn367) in the carboxy terminus of the protein (19). This suggested that the SHBG in PCT cell extracts is incompletely or differentially glycosylated as compared with the secreted protein.

From previous studies, we know that when both N-glycosylation sites on SHBG are disrupted, *i.e.* in $SHBG^{+OG/-NG}$ (Fig. 1A), its electrophoretic microheterogeneity is lost and its apparent size is reduced consistent with the loss of the two N-linked oligosaccharide chains (19). We therefore reexamined the electrophoretic mobilities of SHBG in the PCT cell medium and cell extracts before and after treatment with *N*-glycosidase F (Fig. 1C). When the N-linked oligosaccharides were removed in this way from the SHBG in the PCT culture medium, the electrophoretic microheterogeneity was lost and the apparent size was reduced as expected (Fig. 1C). However, although the electrophoretic microheterogeneity and apparent molecular size of SHBG in the PCT cell extracts are also reduced by treatment with *N*-glycosidase F, it was obvious that its apparent molecular size was slightly smaller than the *N*-glycosidase F-treated SHBG from PCT cell medium (Fig. 1C). This suggests that, unlike the SHBG in the PCT cell medium, the SHBG in PCT cell extracts lacks the O-linked oligosaccharide at Thr7.

To test this, we stably transfected PCT cells with expression vectors that constitutively express either a SHBG mutant lacking the two N-glycosylation sites ($SHBG^{+OG/-NG}$) or a mutant in which all three glycosylation site were disrupted ($SHBG^{-OG/-NG}$), as shown in Fig. 1A, and then compared the electrophoretic properties of the SHBG in cell culture medium and in the cell extracts (Fig. 1D). The difference in apparent molecular sizes of the two mutant proteins in the culture medium is consistent with the presence of an O-linked oligosaccharide at Thr7, and a molecular size of 40 kDa for the SHBG polypeptide (20). Moreover, whereas the $SHBG^{+OG/-NG}$ mutant appears to be O-glycosylated in the culture medium, most of the $SHBG^{+OG/-NG}$ mutant in the corresponding PCT cell extract appears to be unglycosylated because it migrates with the same electrophoretic mobility as the $SHBG^{-OG/-NG}$ mutant, which lacks all three glycosylation sites (Fig. 1D).

Intracellular SHBG enhances androgen uptake and accentuates androgen-dependent *Kap* expression in PCT cells

The SHBG that accumulates within the epithelial cells of the PCTs could sequester free steroids from the glomerular filtrate, and either enhance or block their actions at the nuclear hormone receptor level. To measure the cellular uptake of an SHBG steroid ligand, similar numbers (2×10^5) of parental PCT and PCT-SHBG cells were incubated with [^{3}H]DHT for increasing time points up to 6 h after a change of culture medium (Fig. 2A). The change of medium just before the experiment was done to

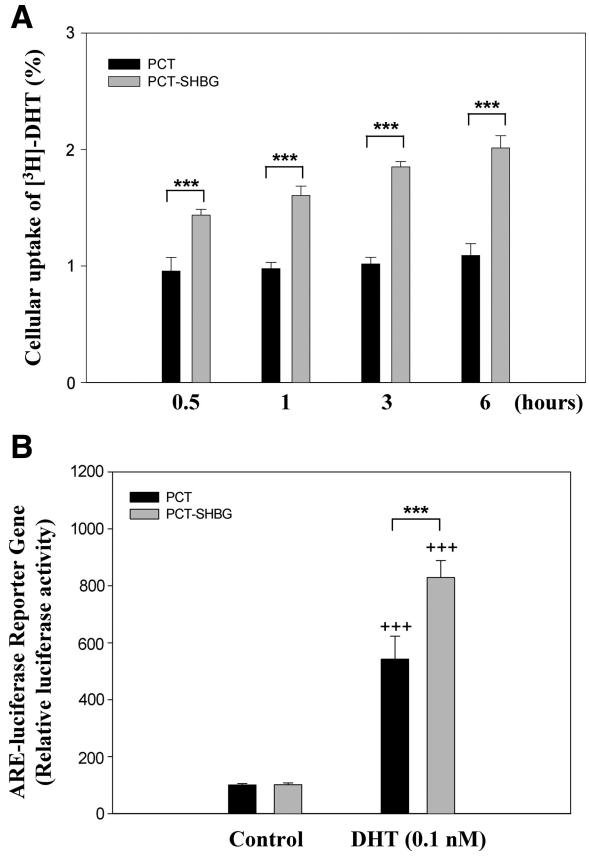

FIG. 2. Influence of intracellular SHBG on cellular androgen uptake and response. A, To measure the intracellular uptake of sex steroid hormone, PCT and PCT-SHBG cells were incubated with 3 nM [^{3}H]DHT for increasing times. The intracellular accumulation of [^{3}H]DHT in these cells was measured at each time point at least in triplicate and expressed as a percentage of the total amount of [^{3}H]DHT in cell culture medium. The experiment was repeated three times, and the cumulative values are expressed as means ± SD. ***, $P < 0.001$ at each time point. B, PCT and PCT-SHBG cells were transfected with an ARE reporter gene construct and then treated with 0.1 nM DHT for 18 h. The culture medium was replaced with fresh medium containing 0.1 nM DHT, and the cells were incubated for a further 6 h. Cell lysates were collected for luciferase activity and β-galactosidase activity as a control for transfection efficiency. The values represent means ± SD. The DHT treatment significantly increased ARE reporter gene activity in both PCT and PCT-SHBG cells when compared with vehicle-treated cells (+++, $P < 0.001$), and the ARE reporter gene activity was also significantly higher in the PCT-SHBG cells than the PCT cells after DHT treatment (***, $P < 0.001$).

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minimize any effect that secreted SHBG in the medium might have on steroid uptake. In this experiment, the accumulation of [^{3}H]DHT by PCT-SHBG cells already exceeded that observed in PCT cells by 30 min (1.5-fold *vs.* PCT) and increased progressively at 1 h (1.6-fold *vs.* PCT), 3 h (1.8-fold *vs.* PCT) and 6 h (1.9-fold *vs.* PCT). Moreover, whereas the relative amount (%) of [^{3}H]DHT that accumulated within the PCT cells from the culture medium did not increase over time, the relative amounts of [^{3}H]DHT sequestered by PCT-SHBG cells increased progressively at each time point (Fig. 2A).

To assess whether enhanced DHT uptake by PCT-SHBG cells influences AR-mediated actions, we first transiently introduced an androgen response element-luciferase (ARE-luc) reporter gene into PCT and PCT-SHBG cells, and then treated them with 0.1 nM DHT or vehicle control for 24 h. We observed robust (5.4-fold and 8.1-fold) increases ($P < 0.001$) in ARE-luc reporter gene activity in the DHT-treated PCT and PCT-SHBG cells, respectively, as compared with the corresponding vehicle-treated control cells (Fig. 2B), and the response was significantly ($P < 0.001$) greater (1.5-fold) in the PCT-SHBG cells than in PCT cells (Fig. 2B).

As PCT cells express the endogenous murine *Kap* gene, and because human *SHBG* transgenes are expressed in the same cell types that express *Kap* within the PCTs (5), we assessed how the presence of human SHBG within PCT cells might influence the basal expression of *Kap* in these cells. To accomplish this, we first cultured PCT and PCT-SHBG cells in medium depleted of steroids [*i.e.* containing 2% dextran charcoal-treated fetal bovine serum (FBS)], and then treated them for 5 d in the same medium with or without 10 nM DHT or estradiol (E2). The Kap mRNA levels were determined in the cells after this treatment in relation to mouse glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA levels, and it was apparent that the basal expression of *Kap* in the absence of steroids was already very much higher (22.1-fold) in the PCT-SHBG *vs.* PCT cells after vehicle-alone (control) treatments (Fig. 3A). The *Kap* gene responded robustly to the DHT treatment in both cell types, as assessed by an increase in the relative abundance of Kap mRNA, but responded only in the PCT-SHBG cells after treatment with E2 (Fig. 3A). It should also be noted that the magnitude of the response to DHT in terms of the actual increase in Kap mRNA level was much greater in the PCT-SHBG cells than in the PCT cells (Fig. 3A).

To examine whether the induction of *Kap* gene expression in the PCT-SHBG is due to higher AR activity or an AR-independent effect of intracellular SHBG, we conducted a separate set of experiments in which PCT and PCT-SHBG cells were incubated in medium containing

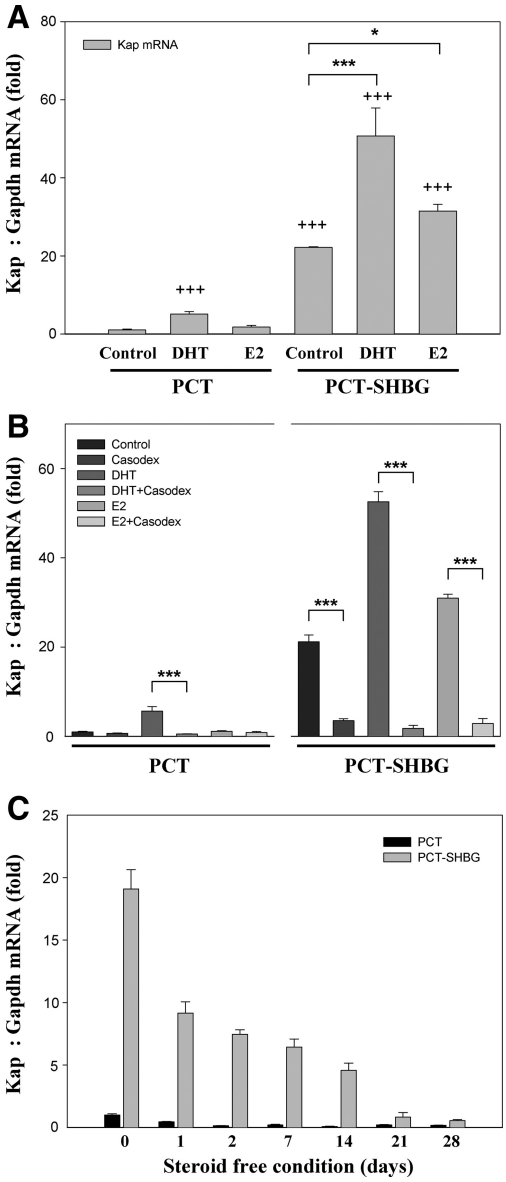

FIG. 3. Influence of intracellular SHBG on murine *Kap* gene expression after sex steroid treatment and withdrawal. A, PCT or PCT-SHBG cells were treated with vehicle alone (control) or with DHT or E2, and Kap mRNA levels were determined by quantitative RT-PCR. Cells were grown in phenol red free DMEM/F12 medium supplemented with 2% dextran charcoal-treated FBS for 5 d before daily treatments with steroids at 10 nM. Gapdh mRNA was used as in internal control. The values represent means ± SD. +++, $P < 0.001$ *vs.* PCT vehicle-alone (control)-treated cells. ***, $P < 0.001$; *, $P < 0.05$ *vs.* PCT-SHBG vehicle-alone (control)-treated cells. B, PCT or PCT-SHBG cells were incubated in medium containing 10 nM DHT or E2 with or without 10 μM Casodex (bicalutamide) for 5 d, and Kap mRNA levels were determined by quantitative RT-PCR. Gapdh mRNA was used as in internal control. The values represent means ± SD. Significant difference is ***, $P < 0.001$ when PCT or PCT-SHBG cells are compared with antiandrogen (Casodex) plus hormone *vs.* hormone alone. C, PCT and PCT-SHBG cells were cultured in DMEM/F12 medium supplemented with 2% FBS for 24 h before substitution with phenol red free DMEM/F12 medium supplemented with 2% dextran charcoal-treated FBS for up to 28 d. Kap mRNA levels of PCT or PCT-SHBG cells were determined by quantitative RT-PCR, and Gapdh mRNA was used as an internal control. The values represent means ± SD.

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10 nM DHT or E2 with or without 10 μM Casodex (bicalutamide), an AR antagonist, for 5 d with daily treatments. The results revealed that Kap mRNA levels in PCT-SHBG cells were no longer markedly elevated after treatment with Casodex alone (Fig. 3B) and that DHT-stimulated increases in Kap mRNA levels in PCT and PCT-SHBG cells were almost completely blocked by Casodex (Fig. 3B). In addition, cotreatment with Casodex also blocks the E2-stimulated increases in Kap mRNA level in PCT-SHBG cells, indicating that this stimulation must also be mediated by the AR (Fig. 3B), and this is consistent with reports that E2 is capable of activating the AR in mouse kidney (21) and prostate cancer cells (22).

The enhanced ability of PCT-SHBG cells to sequester DHT from the culture medium, and the substantial difference in the relative abundance of Kap mRNA between the PCT and PCT-SHBG cells after 5 d of culture in the absence of steroid, together suggested that the SHBG within the PCT cells retains steroids for a prolonged period of time. To test this, we compared the Kap mRNA levels in PCT and PCT-SHBG cells over time (0–28 d) after transferring them from culture medium containing 2% FBS (*i.e.* in the presence of steroid hormones and their precursors) to culture medium containing 2% dextran charcoal-treated FBS (*i.e.* in the absence of steroids). When the cells were grown in the presence of steroids (*i.e.* at the zero time point), this again demonstrated that *Kap* expression in PCT-SHBG cells is very much greater (~19 fold) than in PCT cells, based on quantitative RT-PCR measurements of Kap mRNA levels in relation to the Gapdh mRNA control (Fig. 3C). More importantly, the Kap mRNA levels in the PCT cells dropped to base line levels within 2 d of culture in steroid-free medium (Fig. 3C), whereas the Kap mRNA levels in PCT-SHBG cells decrease by about 50% within 24 h after steroids were withdrawn and remain at approximately the same level for about 1 wk before slowly decreasing to levels approaching those in PCT cells by 4 wk of culture in steroid-free medium (Fig. 3C).

To further demonstrate that the steroid-binding properties of the intracellular SHBG in PCT-SHBG cells are responsible for the increase in endogenous *Kap* gene expression, we produced two other PCT cell lines that overexpressed human SHBG mutants with either reduced (PCT-SHBG S42A) or negligible (PCT-SHBG S42L) steroid-binding activity (23). Whereas these SHBG mutants are expressed at similar levels as the fully-functional wild-type SHBG in PCT cells (Fig. 4, A and B), the Kap mRNA levels in PCT cells expressing the SHBG S42A mutant were 60% of those in cells expressing the wild-type SHBG ($P < 0.05$), and in cells expressing SHBG S42L they were the same as in the parental PCT cells, and only 6% of those in cells expressing the wild-type SHBG (Fig. 4C).

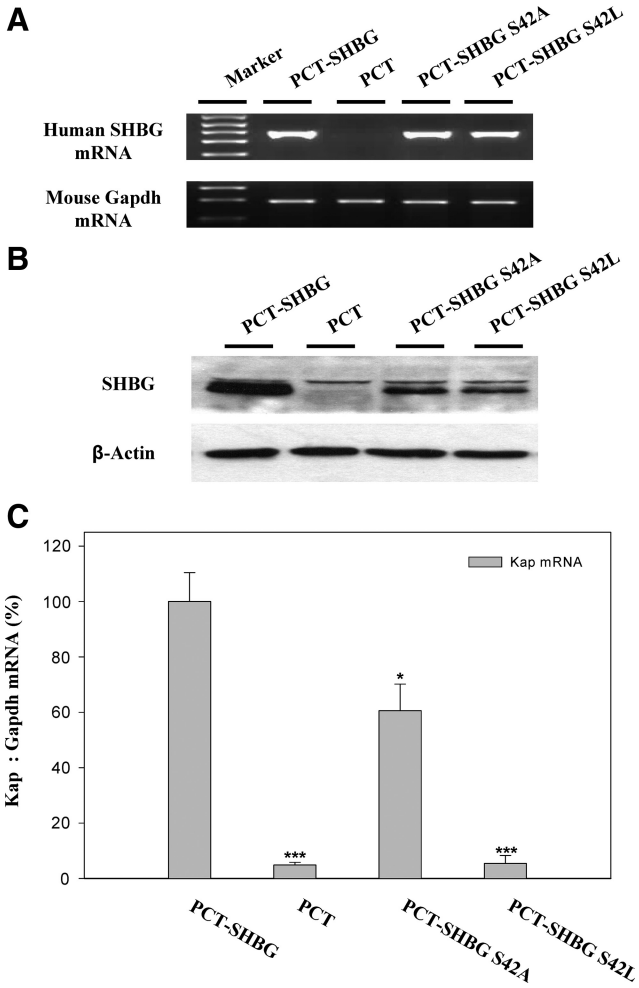

FIG. 4. Influence of wild-type human SHBG and SHBG variants with reduced affinities for steroids on murine *Kap* gene expression in PCT cells. A, human SHBG mRNA is detected by RT-PCR in PCT cells after transfection with expression constructs for wild-type SHBG or SHBG mutants with reduced (SHBG S42A) or no detectable (SHBG S42L) steroid-binding activity, and Gapdh mRNA was measured as an internal control. B, Presence of human SHBG in PCT cell extracts was assessed by Western blotting using the 11F11 monoclonal antihuman SHBG antibody *vs.* β-actin as an internal control. C, Kap mRNA levels of PCT cells grown in DMEM/F12 medium supplemented with 2% FBS were evaluated by quantitative RT-PCR, and Gapdh mRNA was used as an internal control. The values represent means ± SD. ***, $P < 0.001$; *, $P < 0.05$ *vs.* PCT-SHBG cells.

Identification of other androgen-regulated genes the expression of which is either enhanced or suppressed by the presence of SHBG in PCT cells

To identify other androgen-responsive genes that are influenced by the presence of SHBG in PCT cells, we performed gene expression profiling using total RNA from PCT-SHBG and PCT-SHBG S42L cells, which had been cultured in phenol red-free DMEM/F12 medium supplemented with 2% dextran charcoal-treated FBS for 72 h after exposure to testosterone (100 nM) or DHT (100

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nM) for 24 h. This microarray analysis defined 125 transcripts, the levels of which were increased, and 277 transcripts, the levels of which were reduced, by exposure to testosterone by >1.7-fold (Supplemental Table 1 published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org); GEO accession number GSE26058. In addition, we also identified 176 transcripts the levels of which were increased and 231 transcripts the levels of which were decreased after DHT treatment by >1.7-fold (Supplemental Table 1). Among these androgen-regulated genes, 95 were up-regulated and 175 were down-regulated by more than 1.7-fold by both testosterone and DHT.

Four up-regulated and two down-regulated genes were selected for further analysis in PCT, PCT-SHBG, and PCT-SHBG S42L cells pretreated with testosterone or DHT and then cultured for 3 d in the absence of steroid, as described above. When quantitative RT-PCR measurements of specific transcripts in PCT-SHBG cells were compared with those in PCT or PCT-SHBG S42L cells, four genes (*Adh7*, alcohol dehydrogenase 7; *Vcam1*, vascular cell adhesion molecule-1; *Areg*, amphiregulin; *Tnfaip2*, TNFα-induced protein 2) were highly induced in the PCT-SHBG cells ($P < 0.001$ *vs.* either PCT or PCT-SHBG S42L cells), irrespective of their treatment with testosterone or DHT (Fig. 5, A and B). Although the relative levels of Tnfaip2 mRNA were higher (2-fold) in testosterone-treated PCT-SHBG S42L cells than in PCT cells (Fig. 5A), this was not observed in the DHT-treated cells (Fig. 5B).

Interestingly, the two androgen down-regulated genes we studied further, *i.e.* the claudin-2 (*Cldn2*) and odd-skipped related 2 (*Osr2*) genes, were significantly down-regulated ($P < 0.05$ to $P < 0.001$) by testosterone or DHT pretreatment in PCT-SHBG cells, when compared with PCT or PCT-SHBG S42L cells in which Cldn2 and Osr2 mRNA levels were not significantly different after treatment with these androgens (Fig. 5, A and B).

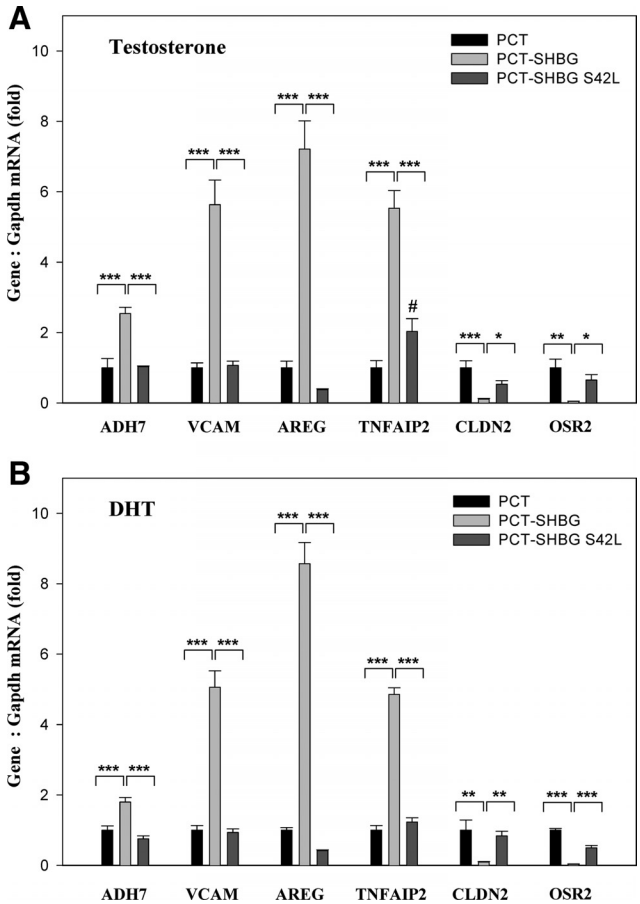

FIG. 5. Up-regulation and down-regulation of androgen-responsive genes is accentuated by the presence of functional SHBG in PCT cells after treatment with testosterone (A) or DHT (B). PCT, PCT-SHBG, and PCT SHBG S42L cells were treated with testosterone (100 nM) or DHT (100 nM) for 24 h before substitution with phenol red free DMEM/F12 medium supplemented with 2% dextran charcoal-treated FBS for 72 h. The gene expression profiles were assessed in the androgen-treated PCT-SHBG *vs.* PCT-S42L cells using an Illumina MouseWG-6, version 2.0 expression bead chip (see Supplemental Table 1), and examples of the most highly up-regulated (*Adh7*, alcohol dehydrogenase 7; *Vcam1*, vascular cell adhesion molecule-1; *Areg*, amphiregulin; *Tnfaip2*, TNFα-induced protein 2) and down-regulated (*Cldn2*, claudin 2; *Osr2*, odd-skipped related 2) genes, were selected for analysis of changes in their mRNA levels by quantitative RT-PCR, using Gapdh mRNA as an internal control. The values of gene-specific mRNA-Gapdh mRNA ratios are shown as fold changes when compared with the mean values in parental PCT cells which were assigned a value of 1. Significant differences are ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ when PCT-SHBG cells are compared with parental PCT cells, and PCT-SHBG cells are compared with PCT-SHBG S42L cells. Note that the values in parental PCT and PCT-SHBG S42L cells are not different, apart from the significantly higher Tnfaip2 to Gapdh mRNA ratio in PCT-SHBG S42L cells (#, $P < 0.05$).

Intracellular SHBG modulates the nuclear retention of the AR in PCT cells

To determine whether the sequestration of androgen by intracellular SHBG alters the stability and/or nuclear retention of the AR, we performed an experiment in which the nuclear AR-cytoplasmic AR ratio was determined in PCT, PCT-SHBG, and PCT-SHBG S42L cells. To accomplish this, the cells were transfected with a human AR expression vector and maintained for 6 h in the presence of 10 nM DHT and then cultured for 24 h in medium containing 2% dextran charcoal-treated FBS (Fig. 6). This was done because endogenous levels of the murine AR in PCT cells are too low to be reliably detected by immunohistochemistry. Although these cells all contain similar amounts of human AR mRNA (Fig. 6A), the relative amounts of the AR were consistently higher in PCT-SHBG cells when compared with either PCT or PCT-SHBG S42L cells, and the nuclear AR-cytoplasmic AR ratio in PCT-SHBG was significantly ($P < 0.01$) higher than in either parental PCT or PCT-SHBG S42L cells, when cultured for 24 h under these conditions in steroid-free medium (Fig. 6B).

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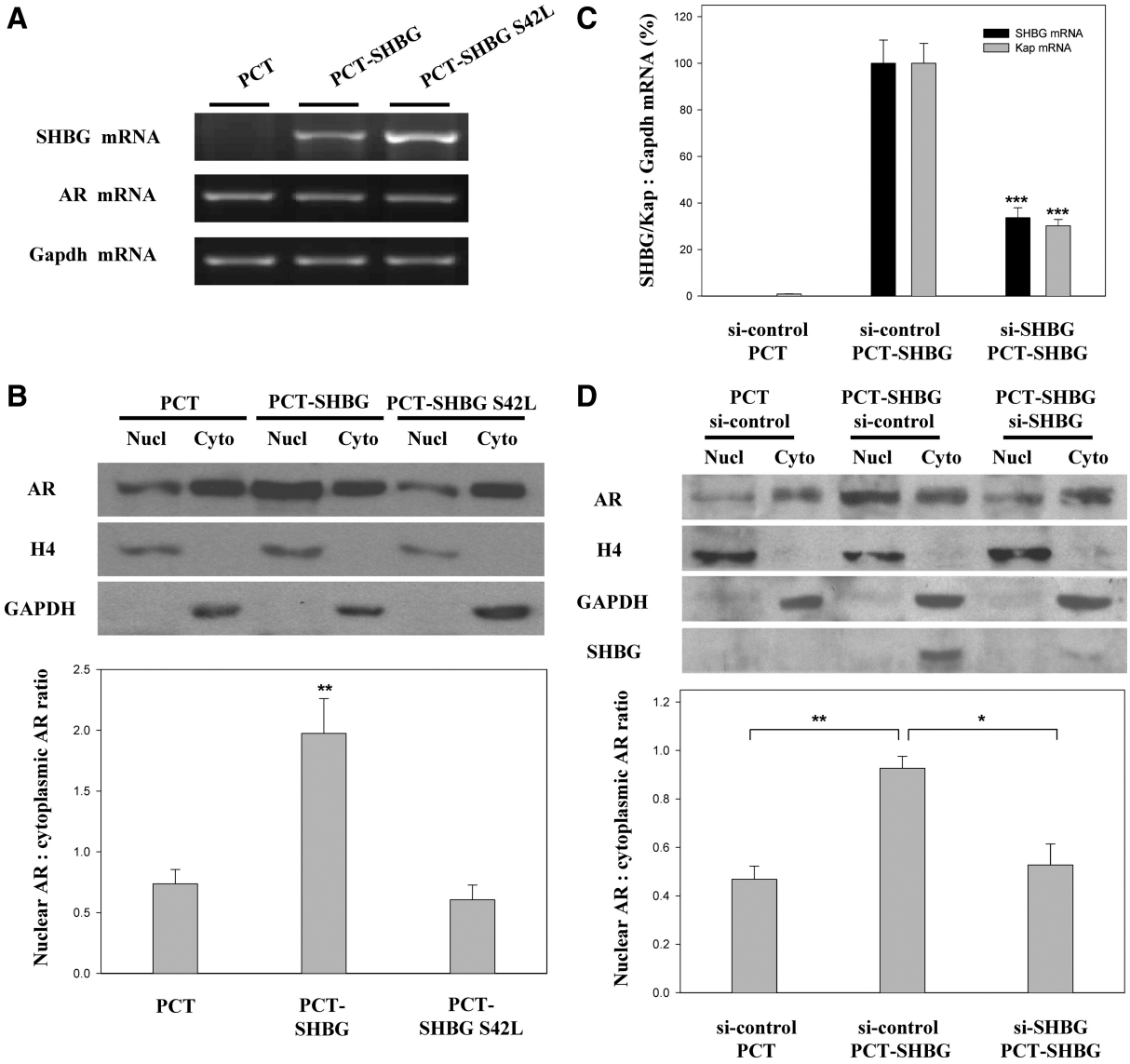

FIG. 6. Intracellular SHBG influences the cellular localization of the AR. In panels A and B, PCT cells and PCT cells expressing either wild-type SHBG or SHBG S42L were transiently transfected with a human AR expression vector and treated with 10 nM DHT for 6 h and then cultured in medium containing 2% dextran charcoal-treated FBS for a further 24 h. In panel A, RT-PCR was used to detect the wild-type or mutant human SHBG mRNAs, the human AR mRNA, and mouse Gapdh mRNA as an internal control. In panel B, the levels of human AR in the corresponding nuclear (nucl) and cytoplasmic (cyto) extracts were assessed by Western blotting. For these experiments, 40-μg samples of nuclear and cytoplasmic protein extracts were analyzed, and histone H4 (nuclear protein) and GAPDH (cytoplasmic protein) were also examined as internal subcellular controls and were used to normalize AR levels in the nuclear and cytoplasmic extracts, respectively. Western blots for AR, histone H4 (H4), and GAPDH in a representative experiment are shown. This experiment was repeated four times, and the mean ± SEM nuclear AR-cytoplasmic AR ratios are shown. **, $P < 0.01$ when PCT-SHBG cells are compared with PCT or PCT SHBG S42L cells. In panels C and D, PCT cells and PCT-SHBG cells were transfected with either 100 nM control siRNA (si-control) or SHBG siRNA (si-SHBG), treated with 10 nM DHT for 6 h, and then cultured in medium containing 2% dextran charcoal-treated FBS for a further 24 h. In panel C, SHBG and Kap mRNA levels were determined by quantitative RT-PCR, using Gapdh mRNA as an internal control. The values represent means ± SD of four experiments. ***, $P < 0.001$ when si-SHBG-treated PCT-SHBG cells are compared with si-control-treated PCT-SHBG cells. In panel D, PCT or PCT-SHBG cells were transiently transfected with a human AR expression vector together with si-RNAs and treated with 10 nM DHT for 6 h, before being cultured in medium containing 2% dextran charcoal-treated FBS for a further 24 h. The levels of AR in samples (40 μg) of nuclear and cytoplasmic extracts were assessed by Western blotting in three separate experiments, as described in panel B above. In addition, a Western blot of SHBG in the cytoplasmic extracts was performed to assess the extent of SHBG siRNA-mediated SHBG depletion. The mean ± SEM nuclear AR-cytoplasmic AR ratios were calculated in PCT-SHBG cells treated with the control si-RNA or SHBG siRNA and in PCT cells treated with control si-RNA. **, $P < 0.01$ when si-control PCT-SHBG cells are compared with si-control- treated PCT cells. *, $P < 0.05$ when SHBG siRNA-treated PCT-SHBG cells are compared with si-SHBG-treated PCT-SHBG cells.

To examine whether the presence of a functional SHBG within the PCT cells is directly responsible for enhancing the nuclear accumulation of the AR, we depleted SHBG levels in PCT-SHBG cells by treating them with a small interfering RNA (siRNA) specific for SHBG mRNA (Fig. 6C). In addition to reducing the SHBG mRNA and

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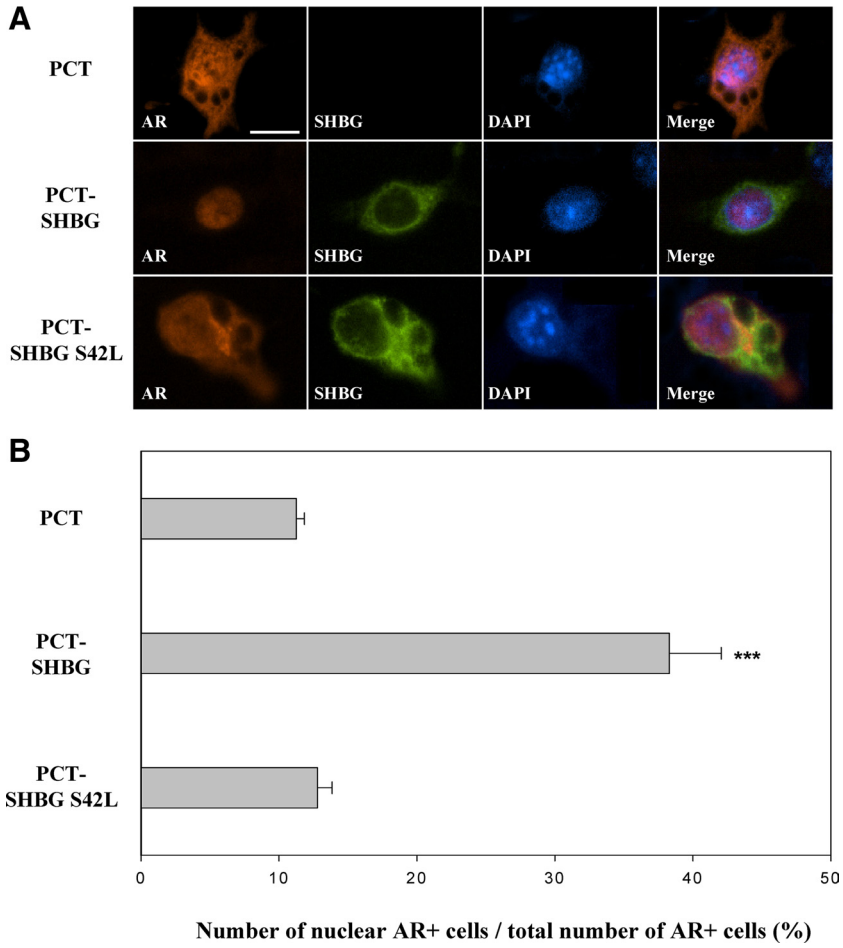

FIG. 7. Nuclear localization of the AR is enhanced in PCT cells by the presence of functional SHBG in the cytoplasm. A, PCT cells were transiently transfected with a human AR expression vector and incubated with medium depleted of steroids for 48 h. Cellular human AR and SHBG was localized at ×400 magnification by immunofluorescent staining with rabbit antihuman AR (*red*) antibody and 11F11 mouse monoclonal antihuman SHBG (*green*) antibody, respectively. Cell nuclei were identified by 4′,6-diamidino-2-phenylindole (DAPI) staining (*blue*). The nuclear localization of the AR was evaluated by merging individual images. B, The number of cells containing nuclei that were AR positive (AR+) was counted and compared as a percentage of the total number of AR+ cells (nuclear and/or cytoplasmic staining) within multiple fields. The values represent means ± SD. ***, $P < 0.001$ *vs.* PCT or PCT SHBG S42L. *Scale bar*, 10 μm.

intracellular SHBG levels, the SHBG siRNA treatment reduced androgen-stimulated Kap mRNA levels in these cells (Fig. 6C), which is consistent with the involvement of SHBG in maintaining higher basal Kap mRNA levels in PCT-SHBG cells, as compared with PCT cells (Fig. 3A). As expected, when PCT-SHBG cells were transfected with a human AR expression vector together with a control siRNA, their nuclear AR-cytoplasmic AR ratio was significantly increased ($P < 0.01$) when compared with that observed in PCT cells treated in the same way (Fig. 6D). Most importantly, the nuclear AR-cytoplasmic AR ratio in PCT-SHBG transfected with a human AR expression vector together with a SHBG siRNA was significantly lower ($P < 0.05$) than in the PCT-SHBG cells treated with the control siRNA (Fig. 6D).

The relationship between the nuclear localization of the AR and the presence of SHBG within PCT cells was further evaluated after a more prolonged withdrawal of steroids. To accomplish this, we first cultured PCT cells, PCT-SHBG cells, and PCT-SHBG S42L cells in medium containing 2% FBS for several days before transfecting them with the human AR expression plasmid, and we then incubated them with medium containing 2% dextran charcoal-treated FBS for 2 d. We then compared the percentage of cells in which the AR was predominantly in the nucleus *vs.* the cytoplasm across the three different cell types (Fig. 7), and this revealed that approximately 40% of the PCT-SHBG cells have the AR in the nucleus, whereas only approximately 12% of the PCT and PCT-SHBG S42L cells have AR in their nuclei (Fig. 7), and this difference was significant ($P < 0.001$).

Discussion

In mice that express human *SHBG* transgenes, we have observed immunoreactive human SHBG within several steroid target tissues and specific cell types (5, 24, 25). We have shown previously that human SHBG can be sequestered from the blood into the stromal matrix of the uterine endometrium, and that this is mediated by a steroid ligand-dependent interaction between SHBG and the C-terminal domains of two members of fibulin family (fibulin1D and fibulin2) of matrix-associated proteins (24). In contrast to the uterus, which does not express human *SHBG* transgenes in these mice (24), we have found that some specific epithelial cell types, including those in specific segments of renal PCTs (5) retain human SHBG within their cytoplasm. This is remarkable because the human SHBG mRNA in these cell types comprises the same complement of exonic sequences encoding the SHBG precursor polypeptide, which is normally destined for processing into the mature form of SHBG that is secreted into the blood.

The presence of SHBG within specific epithelial cells is not unprecedented because others have noted that SHBG

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accumulates within some human epithelial cell types, including a very early observation in human MCF7 breast cancer cells (26), and the more recent report that human prostate epithelial cells and several human prostate cancer cell lines contain *SHBG* transcripts and immunoreactive SHBG in their cytoplasm (27). Although these latter observations are interesting, they fail to address the obvious questions of whether the presence of SHBG within cells reflects its endocytotic uptake, as reported in early studies of MCF7 cells (28) or more recently via an interaction with the endocytotic receptor, megalin (29), or whether the SHBG within the cells restricts or enhances the actions of its natural steroid ligands. We have sought to address these questions in the studies described in this report.

In preliminary studies (data not shown), we first confirmed the presence of human SHBG within the epithelial cells of the S1/S2 segments of the PCTs, and further demonstrated that the same cell types express high levels of the *Kap* gene in mice. Our transgenic mice are ideal for the latter studies because there is no question that the immunoreactive human SHBG in the transgenic mouse tissues is specific as tissues from the corresponding tissues of wild-type littermates are uniformly negative (5). However, because of the potential contamination of tissues with SHBG from the blood circulation, and because the mouse *Kap* gene is also expressed in the S3 segment of the proximal tubules under the control of thyroid hormone, we have used a murine cell line that retains the characteristics of the same S1/S2 PCT epithelial cell types in which the human SHBG accumulates *in vivo*. As in our transgenic mice, a proportion of the SHBG produced by these cells is secreted, but a significant amount is retained within the cytoplasm of the cells.

The immediate question was whether these cells first secrete human SHBG and then re-internalize it via some type of endocytotic receptor-mediated event. We tested this by growing untransfected PCT cells in conditioned medium from CHO cells that express high levels of SHBG, then harvested them for analysis of SHBG in cytosolic extracts, and failed to find any evidence of a cell uptake of SHBG (data not shown). This is not surprising because the cells were grown in the presence of 2% FBS, and the levels of SHBG in the medium are very much lower than those of other plasma proteins, many of which are ligands for endocytotic receptors, such as megalin (30). We therefore conclude that reinternalization of secreted SHBG does not occur in PCT cells, and our biochemical characterization of the human SHBG extracted from PCT cells supports this assumption. In particular, the glycosylation status of the SHBG retained by the PCT cells is quite different from that associated with the SHBG secreted by the cells and is indicative of incomplete or stalled glycosylation of the protein within the endoplasmic reticulum. In support of this, the intracellular SHBG extracted from PCT cells is abnormally N-glycosylated, and does not appear to be O-linked glycosylated at Thr7. The latter is important because O-linked glycosylation occurs during the final stages of glycoprotein modification within the Golgi and is a hallmark of the secreted form of SHBG (31). It is therefore likely that SHBG produced in PCT cells is trapped or held up within the Golgi apparatus, and incomplete glycosylation and a failure to add an O-linked carbohydrate at Thr7 may interfere with the proper intracellular sorting of the protein, as reported for the interleukin-2 receptor (32), and/or possible interactions with putative cargo receptors (33) or proteins important for vesicular trafficking. This does not appear to be a cell-specific phenomenon because we have observed intracellular accumulations of the same type of incompletely glycosylated human SHBG after overexpression in other cell lines including HuTu 80 cells and LNCaP cells (Supplemental Fig. 1).

The observation that SHBG accumulates within specialized epithelial cells of the kidney that are targets of androgen action suggests a novel function of SHBG which extends well beyond its role as a plasma steroid transport protein. Although our studies have relied primarily on the use of a murine kidney cell line for studies of human *SHBG* expression and function, the *SHBG* gene is also expressed in the human fetal kidney and small intestine (G. L. H. unpublished data). Thus, the production and accumulation of SHBG within PCT epithelial cells could serve to sequester small amounts of free steroids within the glomerular filtrate, rather than from the blood circulation, and control the access of these active androgens to the AR. Intuitively, one might suspect that the SHBG within these cells would limit the actions of androgens, because this is generally considered to be the main function of SHBG within the blood circulation, but our data all suggest that SHBG within these kidney cell types acts to enhance and prolong the actions of androgens, especially under conditions where the supply of androgens is limited.

In our experiments, the SHBG within the PCT cells not only promotes a net influx of androgen from the culture medium but it also accentuates the acute activation of an ectopically expressed ARE-luc reporter gene, as well as the sustained and very marked activation of the endogenous androgen-responsive *Kap* gene. The latter is blocked by the highly specific AR antagonist Casodex, and the AR must therefore be the key mediator of this effect. Moreover, these actions are clearly dependent of the ability of the intracellular SHBG to bind steroid because they are much reduced in the presence of an SHBG mutant with reduced steroid binding, and are completely lost in PCT cells that express an SHBG with no measurable affinity for androgens.

Gene expression profiles were assessed in an additional experiment in which PCT cells that contain wild-type

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SHBG, or a steroid-binding-deficient (SHBG S42L) SHBG, were pretreated with 100 nM testosterone or DHT, and then withdrawn from steroid for 3 d. This time point was chosen because it represents the point at which the androgen-response of the *Kap* gene is lost after hormone withdrawal in PCT cells that lack SHBG. In this experiment, we observed a remarkable convergence in the degree of up-regulation or down-regulation of androgen-responsive genes after the PCT cells containing the wild-type SHBG were treated with either testosterone or DHT.

Further confirmation of changes in gene expression profiles was made using quantitative RT-PCR, which indicated that a functional SHBG within PCT cells is able to markedly increase or decrease in relative abundance of androgen-responsive genes over that seen in PCT cells lacking SHBG or containing a steroid-binding-deficient SHBG. These murine androgen-responsive genes include *Areg*, which is a heparin-binding epidermal growth factor (EGF) family member that binds to the EGF receptor with high affinity (34). Whether this is a direct effect of androgens on *Areg* expression is debatable because others have suggested that androgen induction of *AREG* expression in human LNCaP cells is due to an indirect effect of androgen on EGF receptor pathways (35). Another gene that was highly induced by androgens in PCT-SHBG cells is *Vcam-1*, which is expressed in endothelial cells (36), as well as other cell types including renal epithelial cells (37). There is no androgen response element (ARE) within the *VCAM-1* promoter, and DHT is thought to enhance *VCAM-1* expression via nuclear factor-κB activation (38, 39). Like *VCAM-1*, *TNFAIP2* transcripts were also originally identified in human endothelial cells (40) and *TNFAIP2* is highly expressed in human fetal kidney tissue (41). Moreover, our studies show that *Tnfaip2* is one of the most highly induced androgen-responsive genes in PCT cells that contain functional SHBG. Although it has not previously been reported that *Adh7* responds to androgen in the mouse kidney, the mouse *Adh1* gene responds to androgens by increased mRNA accumulation and increased catalytic enzyme activity in the kidney (42). In our experiments, *Adh7* expression in PCT cells treated with testosterone or DHT was very significantly enhanced by the presence of SHBG in these cells.

The presence of functional SHBG in PCT cells also accentuates the repressive effects of androgens on many genes in these mouse kidney epithelial cells, *e.g. Cldn2* and *Osr2*. Cldn2 is a member of the tight junction protein family that is highly expressed in S2–S3 segments of the proximal tubule (43, 44), and it is involved in trans-epithelial reabsorption of Na^+, Cl^-, and water in these segments (45). *Osr2* transcripts are highly expressed during embryo developmental stage and encode a zinc finger-containing transcription factor (46). Interestingly, in these and other cases, the effects of both testosterone and DHT were quite similar, and the presence of SHBG influenced their actions similarly as either positive or negative regulators of gene expression, presumably via their actions as ligands of the AR.

We also demonstrate that the enhanced and sustained actions of androgens in PCT cells expressing SHBG can be explained by retention of the AR in the nucleus. These observations are consistent with the concept that intracellular SHBG provides a reservoir of androgen that is available for the AR, and that this acts to promote the nuclear retention and prolonged activation of the AR. This effect could be attributed to a reduced rate of degradation of AR in SHBG-expressing cells grown in the absence of androgens, because the presence of androgens is known to stabilize the AR both *in vivo* and *in vitro* (47, 48), and degradation of the AR is related to its relative affinity for different ligands (49).

In summary, our experiments suggest that the presence of an incompletely glycosylated isoform of human SHBG within an androgen-responsive epithelial cell type not only increases the cellular uptake of its steroid ligands, but it also effectively enhances the activity of the AR by promoting its retention in the nucleus. Most importantly, the stimulation or repression of androgen-responsive genes is enhanced by the presence of intracellular SHBG after steroid withdrawal over prolonged periods of time, and likely up to several weeks. These findings are particularly interesting in light of reports that human prostate epithelial cells express and contain immunoreactive SHBG (27) and recent evidence that prostate cancer cells have the capacity to produce small amounts of androgens locally (50). This therefore raises the obvious question of whether the expression of *SHBG* in prostate cancer cells could contribute to the castration resistant phenotype by accentuating the actions of small amounts of locally produced androgens.

Materials and Methods

Antibodies

Antibodies specific for human SHBG included rabbit antihuman SHBG antibodies (51) and a monoclonal antihuman SHBG antibody (11F11) kindly provided by Dr. J. Lewis (Christchurch, New Zealand). Rabbit anti-AR (C-19, sc-815), goat anti-β-actin antibody (C-11, sc-1615), rabbit anti-histone H4 (sc-8660), and goat anti-GAPDH (V-18, sc-20357) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Expression plasmids and reporter gene constructs

We used the pRC/CMV mammalian expression vector (Life Technologies Corp., Carlsbad, CA) to express cDNAs encoding wild-type human SHBG (18), various glycosylation-deficient mutants (19), or mutants lacking steroid-binding activity (23). We also used an ARE-luc reporter gene constructed in the pGL3-basic vector (Promega Biosciences Inc., San Luis Obispo, CA) and a pSG5 eukaryotic expression vector encoding the human AR (52).

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The small interfering RNA (siRNA) experiments were performed using Lipofectamine RNAi MAX Transfection Reagent (Life Technologies) together with a control siRNA (D-001810-10) or a SHBG siRNA (On-TARGET plus SMARTpool L-014191-00-0005) obtained from Thermo Fisher Scientific Dharmacon Products (Lafayette, CO).

Cell culture

All cell culture reagents were from Life Technologies. The PKSV-PCT cell line was kindly provided by Dr. Anna Meseguer (Barcelona, Spain) with permission from Dr. A. Vandewalle (Paris, France) and grown at 37 C in a 5% CO_2 atmosphere in DMEM/F12 medium supplemented with 2% fetal bovine serum (FBS), insulin (5 μg/ml), dexamethasone (5×10^{-8} M), selenium (60 nM), transferrin (5 μg/ml), T_3 (5×10^{-8} M), EGF (10 ng/ml), D-glucose (20 mM), penicillin (100 U/mol), and streptomycin (100 μg/ml). To generate PCT cells that constitutively express wild-type human SHBG or human SHBG mutants deficient in steroid binding, we transfected PKSV-PCT cells with various cDNA constructs within pRC/CMV, as previously reported for CHO cells (18, 19, 23). Cell lines expressing human SHBG (PCT-SHBG) or human SHBG mutants with reduced (PCT-SHBG S42A) or no detectable (PCT-SHBG S42L) steroid-binding activity were obtained by limiting dilution cloning and screening the culture medium for secreted SHBG using an ultrasensitive time-resolved fluorescence immunoassay (53). In parallel, cloned parental PCT cells that contain a nonfunctional pRC/CMV expression plasmid were used as negative controls.

Assays of androgen uptake by PCT cells *vs.* human SHBG-expressing PCT cells

The abilities of PCT and PCT-SHBG cells to uptake androgens were compared using [^{3}H]5α-dihydrotestosterone ([^{3}H]DHT) because it is the preferred ligand of SHBG. To accomplish this, cell lines were incubated with 3 nM [^{3}H]DHT (GE Healthcare Life Sciences, Baie d'Urfé, Quebec, Canada; specific activity 133.4 Ci/mmol) in 300 μl serum free DMEM/F12 medium at 37 C in a 5% CO_2 atmosphere. Cells were washed twice with Hank's balanced salt solution and harvested with 0.25% trypsin. Cell pellets were resuspended in 0.2 ml ice-cold buffer (0.25 M Tris-HC1, pH 7.5) and mixed with 4 ml scintillation cocktail for radioactivity measurements in a liquid scintillation counter.

ARE-luc reporter gene assay

Briefly, 4×10^5 PCT cells or PCT-SHBG cells were seeded into six-well tissue culture plates 1 d before transfection in 2 ml phenol red-free DMEM/F12 medium (Life Technologies) containing 2% dextran charcoal-treated fetal bovine serum (Thermo Fisher Scientific Inc.). Transient cotransfection of ARE-luc with an AR expression vector, and a pCMVlacZ control plasmid expressing β-galactosidase (β-gal) was performed using Lipofectamine-2000 Transfection Reagent according to the protocol recommended by Life Technologies. For each transfection, the DNA mixture comprised 1 μg of ARE-luc and 0.2 μg of pSG5-AR, and 0.2 μg of pCMVlacZ was incubated for 30 min at room temperature and then applied to the cells. At 24 h after transfection, the cells were treated with 0.1 nM DHT for 18 h and retreated for a further 6 h. After treatment, the cells were washed twice with PBS and harvested by scraping. After centrifugation, cell pellets were resuspended in 100 μl 0.25 M Tris-Cl, pH 7.8, and cells were lysed by three freeze-thaw cycles. Appropriate aliquots of cell extracts were used for measurements of luciferase and β-galactosidase (β-gal) activity. To correct for transfection efficiency, light units from the luciferase assay were divided by the optical density reading from the β-gal assay.

Deglycosylation of human SHBG

A confluent culture of PCT-SHBG cells was subjected to a single freeze/thaw cycle to release SHBG into 50 μl of 0.25 M Tris-HCl (pH 8.0). The cell extracts and medium harvested from the same cells were then treated with *N*-glycosidase F (Roche Diagnostics, Laval, Quebec, Canada) at 37 C for 1 h.

Preparation of nuclear and cytoplasmic extracts

Cells were washed twice with ice-cold PBS and solubilized in hypotonic lysis buffer (10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 1 mM dithiothreitol; and 1 μg/ml protease inhibitor cocktail) at 4 C for 15 min, followed by addition of 10% Nonidet P-40 solution to a final concentration of 0.6% and vortex mixing for 10 sec. Samples were then centrifuged for 30 sec at $10,000 \times g$, and supernatants were transferred to fresh tubes (cytoplasmic fraction). The pellets were washed twice with hypotonic lysis buffer and resuspended in nuclear extraction buffer (20 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 0.42 M NaCl; 0.2 mM EDTA; 1 mM dithiothreitol; 1 μg/ml protease inhibitor cocktail; and 25% glycerol), and vortex mixed for 15 min. After centrifugation for 5 min at $20,000 \times g$, supernatants were transferred to fresh tubes (nuclear fraction).

Western blotting

Cells were washed twice with ice-cold PBS and solubilized in lysis buffer (10 mM Tris, pH 7.5; 150 mM NaCl; 1% Triton X-100; 1 mM phenylmethylsulfonyl fluoride; 0.2 mM sodium orthovanadate; 0.5% Nonidet P-40) at 4 C for 30 min. Nuclear and cytoplasmic protein extracts were prepared as described above. The protein content of cell extracts were determined, and equal amounts were heat denatured in loading buffer and subjected to discontinuous SDS-PAGE with 4 and 10% polyacrylamide in the stacking and resolving gels, respectively. Proteins in the gel were electrotransferred to Hybond ECL nitrocellulose membranes (GE Healthcare Life Sciences). The membranes were blocked for 1 h in PBS containing 0.01% Tween 20 and 5% skim milk, and incubated overnight at 4 C with a primary antibody in the same buffer. The blots were then washed three times in PBS containing 0.01% Tween 20 for 15 min to remove excess antibody, and specific antibody-antigen complexes were identified using horseradish peroxidase-labeled secondary antibody. The ECL (GE Healthcare Life Sciences) was used for detection of antibody-bound complexes, and signals were recorded by exposure to x-ray film.

Immunocytochemistry

Monolayer PCT, PCT-SHBG, or PCT-SHBG S42L cells were grown in standard culture medium as described above and cultured in eight-well chambers on tissue culture glass slides (BD Bioscience, Mississauga, Ontario, Canada). When the cells reached 70% confluence, the cells were washed in PBS and fixed in 100% methanol at −20 C for 20 min. Slides were air dried and then rehydrated in PBS. The fixed cells were blocked with 10% goat serum at room temperature for 30 min and incubated overnight with primary antibodies at 4 C. After this, slides were washed and incubated with Alexa-Fluor-conjugated secondary

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antibodies (Life Technologies) diluted 1:1000 in PBS with 1% normal goat serum at room temperature for 1 h. After washing, slides were mounted in ProLong Gold antifade reagent and examined using a Leica DM4000B fluorescence microscope (Leica Microsystems, Inc., Richmond Hill, Ontario, Canada).

RNA analysis

Total RNA extracts from cells were used to determine human SHBG and AR mRNA levels and mRNAs of various murine androgen-responsive genes. For semiquantitative analyses of human SHBG and AR mRNA, reverse transcription (RT) was performed at 42 C for 50 min using 3 μg of total RNA and 200 U of Superscript II together with an oligo(dT) primer and reagents provided by Life Technologies. An aliquot of the RT product was amplified in a 20-μl reaction using PCR SuperMix (Life Technologies) with oligonucleotide primer pairs corresponding to target mRNA and Gapdh mRNA sequences (Supplemental Table 2). The PCR was performed for 25 cycles at 94 C for 15 sec, 60–65 C for 30 sec, and 72 C for 1 min, and PCR products were resolved by electrophoresis in a 2% agarose gel. For mouse androgen-responsive gene products (see Supplemental Table 2 for oligonucleotide primer sequences), quantitative RT-PCR was carried out in 25 μl containing 12.5 μl of 2 x SYBR Green PCR master mix (Life Technologies), 1 μl each of forward and reverse primers, 2.5 μl of 1:5 diluted RT product, and 8 μl distilled water, and was performed using an ABI Prism 7000 Sequence Detection System (Life Technologies) equipped with a 96-well optical reaction plate. Negative controls, containing water instead of sample cDNA, were used in each plate. All experiments were run in triplicate and mRNA values were calculated based on the cycle threshold and monitored for an amplification curve.

RNA extraction and gene expression profiling

RNA was extracted for gene expression profiling from PCT-SHBG and PCT-SHBG S42L cells using a Nuclospin RNA II kit (Macherey-Nagel GmbH and Co, Düren, Germany). Total RNA (250 ng) was used for *in vitro* transcription-amplification using the Illumina RNA amplification kit (Ambion, Austin, TX), and 1.5 μg of cRNA was hybridized to whole-genome expression array (Mouse WG-6 version 2.0 expression bead chip, Illumina).

Microarray data analysis

The data analysis was performed by using R software (http://www.r-project.org/) and Beadarray software (www.bioconductor.org). Raw intensity values were normalized independently between arrays for each cell type using quantile normalization. The median value of three sample replicates was used to calculate differentially expressed genes. Fold change 1.7 was set as a cut-off value for the differentially expressed genes (GEO accession number GSE26058). P values were produced by t test and are false discovery rate-adjusted (q values).

Statistical analyses

Differences between means were obtained by one-way ANOVA and the Tukey's *post hoc* test using GraphPad Software (GraphPad Inc., San Diego, CA).

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