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Steroid Binding Proteins—Clinical Aspects

EFFECTS OF SEX HORMONE BINDING GLOBULIN (SHBG) ON HUMAN PROSTATIC CARCINOMA

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Summary—The purpose of this study was to determine what effects sex hormone binding globulin (SHBG) might have on the growth and steroid content of human prostate carcinoma. Two human prostate carcinoma cell lines were used for this study, ALVA-41 and ALVA-101. The first part of the study was to determine the effect of SHBG or albumin on the uptake of [^{3}H]DHT in the cells. In this experiment both SHBG and albumin inhibits the uptake of [^{3}H]DHT into each of the cell lines when studied *in vitro*. The degree of inhibition was dependent on the binding capacity of the protein. When [^{3}H]thymidine uptake was measured in each of the cell lines following either the addition of SHBG or albumin to the culture media, an increase in uptake and presumably DNA synthesis was noted in the ALVA-41 and ALVA-101 cells for SHBG additions but not for albumin. Further, this stimulation was increased when testosterone was added to the media, however, [^{3}H]thymidine uptake was decreased by high concentrations of dihydrotestosterone (DHT) or if the SHBG was saturated with DHT prior to being added to the media. The cells also demonstrate high affinity cell membrane receptors for SHBG. Finally, using a 3′, 550 bp cDNA or SHBG, 1.9 and 2.8 kb mRNAs were detected on Northern analysis of the ALVA-101 and ALVA-41 cells. These data indicate SHBG can inhibit uptake of steroids into the prostrate, but also it may act as a stimulus for growth through a SHBG cell surface receptor. In addition, the growth effect may be through an autocrine effect from SHBG or a SHBG-related peptide.

INTRODUCTION

The traditional role of sex hormone binding globulin (SHBG) has been that of a steroid transport protein and determinant of bioavailable sex steroid. However, more recent data suggests that SHBG may have actions on cells in addition to its extracellular transport and steroid binding functions. The initial data from Petra *et al.* [1–7] demonstrated SHBG-like immunoreactivity in a number of steroid responsive tissues including, prostate, endometrium, breast tissue and sperm. Also, SHBG activity was found in the secretions from some of these tissues, specifically breast cyst fluid and seminal fluid [8]. In addition, several early studies using serum fractions enriched with SHBG suggested that, under certain hormonal conditions, increased concentrations of SHBG are associated with increased intracellular transport of sex steroids with high affinity for SHBG [9]. More extensive reviews of this topic have recently been presented [10, 11].

However, the presence of extravascular and cell associated SHBG remained an unexplained phenomenon secondary to the transport and binding functions of SHBG until two laboratories demonstrated specific, high affinity binding sites for SHBG [12, 13]. These high affinity sites (receptors) have been solubilized from membrane fractions and appear to have an M_r of 167,000 daltons [14]. Nakhla *et al.* [15] have also demonstrated that following the interaction of SHBG with its receptor, there is activation of the adenyl cyclase-cyclic AMP membrane signal system. In addition, receptor binding of SHBG is influenced by its association with specific steroid ligands and by alterations in the carbohydrate composition of this glycoprotein [16]. Therefore, SHBG may have a direct function on specific cells outside the vascular compartment.

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However, the biologic function(s) of this interaction is not known.

In the studies presented in this paper, two issues will be examined: (1) The possible physiologic role of membrane binding of SHBG, and (2) the source of the SHBG-like material found in the prostate by immunochemistry. These are not separate issues, but related, in that if a function is determined for SHBG on a particular tissue, it remains to be determined whether it is an endocrine or autocrine mediated function.

MATERIALS AND METHODS

The human prostate cell lines that have been used for these studies are two cell lines initiated at American Lake VA Medical Center. The ALVA-41 and ALVA-101 cell lines were obtained from the bone metastases of men with prostate carcinoma. No other tumors were present in these individuals. The cell lines have androgen receptors. The ALVA-41 and 101 cell lines both demonstrate 5α reductase activity. ALVA–41 cells have demonstrated prostate specific antigen (PSA) by immunohistochemical staining. The ALVA-101 cells demonstrate PSA mRNA following hybridization with an oligonucleotide probe for PSA. Cell lines were cultured in RPMI1640 with 5% fetal calf serum (FCS, Hyclone, Logan, UT) 2 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin–streptomycin (v/v), and 6% CO_2 at 37°C. When experiments were done in FCS-free conditions, the cells were first grown to 60% confluence with FCS, washed, and the media replaced with serum-free RPMI-1640.

The SHBG used in the experiments was prepared from human pregnancy serum. The serum was first precipitated with 48% ammonium sulfate, then put sequentially through Con A-sepharose, DEAE, and testosterone-agarose affinity columns (Prep. 1). For some studies, the androgen affinity column was not used in order to avoid charcoal stripping the material which is necessary to remove the high concentrations of dihydrotestosterone (DHT) saturating the SHBG following elution from the testosterone affinity column, instead of G-200 sepharose column was used following the DEAE step (Prep. 2). The purity of the SHBG was determined by SDS polyacrylamide gel electrophoresis with Coomasie Blue. In Prep. 1, only SHBG bands were noted at 45,000 to 50,000 kDa. In Prep. 2, in addition to the SHBG bands, faint staining was also noted at 30,000 and 67,000 kDa. The androgen affinity material was purified approx. 2000 fold, while Prep. 2 was purified approx. 600 fold. The concentration of SHBG was determined by both DHT saturation analysis and immunoradiometric assay, with materials supplied by Farmos Diagnostica®, Turku, Finland. The protein concentrations of the eluted fraction were determined by the Bradford assay.

Northern analysis of total cytoplasmic RNA was performed using a 550 bp cDNA probe for human SHBG obtained from Dr Geoffrey Hammond. This probe is directed against the 3′ end of the message. Total cytoplasmic RNA was isolated from the cultures by the acid guanidinium isothiocyanate–phenol–chloroform extraction method. RNA concentration was determined by u.v. absorbance at 260 nm. Equal amounts of RNA were denatured at 65°C for 15 min with 2.2 M formaldehyde, and then fractionated by electrophoresis through a 1% agarose–2.2 M formaldehyde gel. The RNA was transferred to a charged nylon membrane filter (GeneScreen, DuPont) overnight using a sponge with standard capillary blotting technique in 10 × SSC (1 × SSC is 0.15 M sodium chloride and 0.015 M sodium citrate). The bound material was covalently crosslinked to the membrane by u.v.-irradiation (Stratalinker, Stratagene). Gel purified DNA fragments were radioactively labeled to a sp. act. of $1–2 \times 10^8$ cpm/μg with [α^{32}P]dCTP (3000 Ci/mmol) by random priming. Filter hybridizations were performed under standard conditions (50% formamide, 5 × SSC, 1% SDS at 42°C). The final post-hybridization wash was with 1.0 × SSC at room temperature. The bound material was visualized by autoradiography, at −70°C using intensifying screens.

Immunocytochemistry for the presence of SHBG was performed using an immuno-affinity purified goat polyclonal antibody prepared against purified human SHBG (courtesy of Dr Philip Petra). The second antibody was an anti-goat FITC labeled antibody (Sigma Chemical Co., St Louis, MO). For this assay cells were grown to confluence in 48 well plates, the media was aspirated and replaced with 1 ml of 2% bovine serum albumin in phosphate buffered saline. After incubating the plates for 1 h at 37°C, the media was aspirated and anti-SHBG antibody was then added and incubated overnight. After aspirating the media the cells were washed × 3 with PBS. 1 ml Of methanol–

acetone was added for 10 min at room temperature. The methanol–acetone mixture was then aspirated, plates were air dried, and 50 μl FITC anti-goat IgG polyclonal antibody (Sigma) was added. After 1 h incubation with the antibody at 35°C the wells were rinsed. Finally, 200 μl FITC mounting fluid was added and the cells observed with a fluorescent microscope.

Receptors for SHBG in the two cell lines utilized for this study have been measured as described previously [14]. Prostatic tumor plasma membranes were prepared by differential centrifugation of tumor nodule homogenate obtained from tumors of the responding cell lines grown in Balb/c athymic mice. Binding assays were done using both enriched plasma membrane fractions and membrane proteins solubilized in CHAPS detergent. Receptor binding was measured using purified [^{125}I]SHBG and displaced with "cold" SHBG. Receptor number and affinity constants were determined using Scatchard analysis.

New DNA synthesis was determined by [^{3}H]thymidine uptake. In this assay tumor cells were plated in 96 well microtiter plates at an initial cell density of 2.5×10^3 cells/well for assays containing 5% FCS in RPMI-1640 or 5×10^3 cells/well for serum-free growth in 100 μl/well. SHBG was diluted in media to a volume of 100 μl and added to the wells such that the total incubation volume was 200 μl. The plates were incubated for 3–6 days at which point 0.1 μCi of [^{3}H]thymidine was added to each well and the incubation continued for 16 h. Cells were then harvested, using a Skatron automatic cell harvester onto glass fiber mats. Tritiated-thymidine incorporation was determined by counting the filters in Packard 1900 CA liquid scintillation counter. Each point is the mean of 6 replicate cultures. Control cultures contained equal concentrations of added human serum albumin (HSA, Sigma) or ovalbumin as protein equivalent controls. The concentration of SHBG added to the culture media ranged from 1–100 ng/well. In experiments where the steroids were removed from the SHBG preparation by stripping with activated charcoal, the preparation was mixed in a 1:1 ratio (v/v) with charcoal and incubated kat 37°C for 2–16 h. Samples were then centrifuged at 3000 **g** for 30 min and the supernatant collected.

The effect of SHBG on cellular uptake of [^{3}H]DHT was assessed by growing cells to confluence, removing the FCS, and replacing it with serum-free media. Following addition of

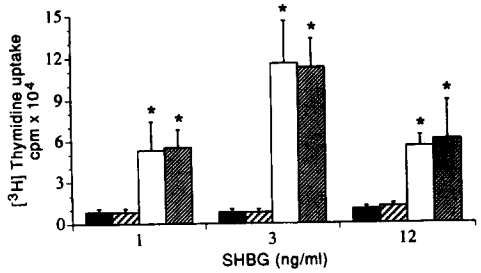

Fig. 1. [^{3}H]thymidine uptake in the ALVA-41 cell line. Albumin—■, albumin + testosterone(T) added—▨, SHBG—□ and SHBG + T added—■. Reported values are mean ± SEM with * = $P < 0.05$.

the serum-free media, 100,000 cpm of [^{3}H]DHT (1,2,6,16,17-[^{3}H]DHT), obtained from New England Nuclear (Wilmington, DE), was added to the media. After 4 h incubation at 37°C, the supernatant was removed and counted in a beta-scintillation counter. The cells were then washed three times with normal saline, trypsinized, lysed and [^{3}H]DHT was counted. Each experiment was performed in triplicate. These studies were done in the basal state, as well as after the addition of HSA, SHBG or SHBG plus a 100-fold excess of cold DHT relative to [^{3}H]DHT.

RESULTS

The initial studies were performed to determine the effects of SHBG on the [^{3}H]thymidine uptake of the two cell lines. As can be seen in Figs 1 and 2, increasing concentrations of SHBG were associated with an increase in DNA synthesis, as assessed by the uptake of [^{3}H]thymidine. Control cultures consisted of equal protein concentrations of HSA added to the wells. No effects on [^{3}H]thymidine uptake was noted in the control cultures. When human pregnancy serum was added in an equivalent protein concentrations to the ALVA-41 cells, the stimulation of [^{3}H]thymidine uptake was

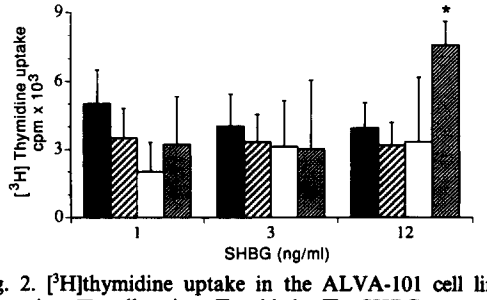

Fig. 2. [^{3}H]thymidine uptake in the ALVA-101 cell line. Albumin—■, albumin + T added—▨, SHBG—□ and SHBG + T added—■. Reported values are mean ± SEM with * = $P < 0.05$.

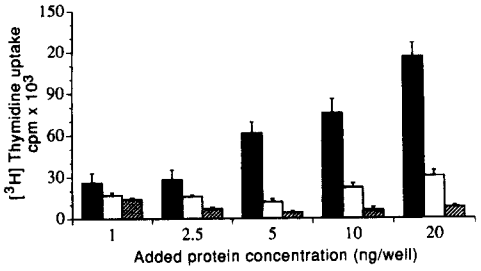

Fig. 3. [³H]thymidine uptake in ALVA-41 cells. SHBG—■, equivalent protein concentration of pregnancy serum—□, equivalent protein concentration of adult male serum—■. Reported values are mean ± SEM.

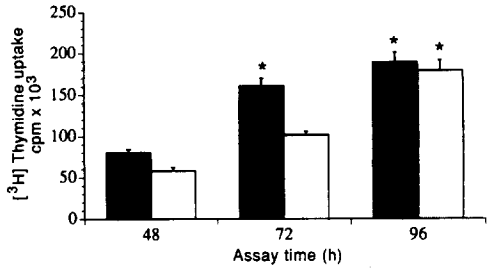

Fig. 5. [³H]thymidine uptake in the ALVA-41 cells. SHBG added—■, 10% FCS—□. Reported values are mean ± SEM with * = $P < 0.05$.

greatest with the SHBG preparation, Fig. 3. The mitogenic response to SHBG was optimal at 48 h and the response was maintained at the peak level up to 72 h.

The effect of the addition of testosterone to the cultures is shown in Figs 1 and 2. No increase in stimulation was noted in the ALVA-41 cells with the addition of 1 nM testosterone. In contrast, the ALVA-101 cells demonstrated a response to the SHBG preparation, but only when testosterone was added to the preparation. No effect from the addition of testosterone to the HSA controls was noted. These findings are consistent with the recently published data that the presence of steroid may be necessary for the full effect of the binding protein on its receptor after initially being added in steroid-free conditions [15]. The reason for the differences between the two cell lines, as far as androgen requirements are concerned, is not clear. At the present time, however, we presume that the ALVA-41 cell line has a maximal response to the SHBG added without testosterone. The effects of androgens were further delineated by stripping steroids from SHBG preparations before addition to the cells. In this case, as is seen in Fig. 4, a decrease in stimulatory activity was observed. Furthermore in the ALVA-101 cells, the activity of the charcoal-stripped SHBG could be returned when testosterone was added to the media. However, recent data also suggests that high concentrations of steroids, when added to the SHBG prior to its addition to cells, will prevent binding to the receptors [11]. This effect was observed in the ALVA-101 cell line, where increasing concentrations of DHT to the SHBG preparation was associated with a loss of activity (data not shown). However, care must be taken in the interpretation of these experiments, since it has been shown that high steroid concentrations themselves can have a deleterious effect on the growth of prostate cells *in vitro* [17].

The time course of the SHBG mitogenic effects are noted in Fig. 5 and are compared to equivalent concentrations of human serum. The greater stimulation with media enriched with SHBG compared to 10% FCS is clearly evident at the 24 and 48 h time points.

Receptor measurements on the cells demonstrated the presence of two classes of receptors. On the ALVA-41 cells, a high-affinity low-capacity receptor with K_d of $1.97 \pm 0.79 \times 10^9\ M^{-1}$ and a binding capacity of 6.51 ± 16.9 fmol/mg protein was found. On the ALVA-101 cells, a high-affinity receptor class was noted with a K_d of $1.32 \pm 0.21 \times 10^9\ M^{-1}$ and a capacity of 59.9 ± 23.6 fmol/mg protein.

The stimulation of cellular proliferation by SHBG may suggest that SHBG binding can

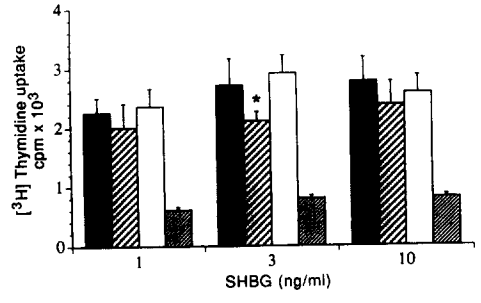

Fig. 4. [³H]thymidine uptake in the ALVA-101 cell line. SHBG with 1 nm added T—■, charcoal stripped SHBG—▨, charcoal stripped SHBG with 1 nm T added—□, albumin protein control—■. Reported values are mean ± SEM with * = $P < 0.05$.

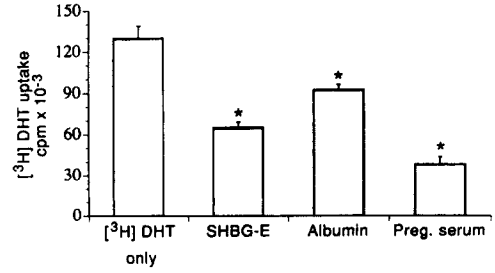

Fig. 6. [³H]DHT uptake in ALVA-101 cells. SHBG-E = SHBG enriched serum. * = $P < 0.05$.

increase the intracellular concentration of androgen. However, as can be seen from the data in Fig. 6, when either SHBG or albumin is added to the media of the cells, inhibition of cellular uptake of steroid occurs. Also, characteristic of the high affinity and low capacity of SHBG for its steroid ligands, the uptake is increased as an excess of cold steroid is added to the SHBG incubation, whereas little change is noted when cold steroid is added to the albumin solution (which would be consistent with the lower affinity and higher capacity of albumin). However, both SHBG and albumin decreased cellular uptake of testosterone and neither quantitatively facilitated entry of testosterone into the cells.

Results of Northern analysis of total cytoplasmic RNA from the ALVA-41 and 101 cell lines are shown in Fig. 7. Two distinct species were identified with the 3′ cDNA probe which migrated to positions corresponding to 1.9 and 3.4 kb. When a 33-mer oligonucleotide probe corresponding to amino acids 48–57 of SHBG (which is encoded for by exon III of the SHBG gene) was used, hybridization was seen only to the 1.9 kb band.

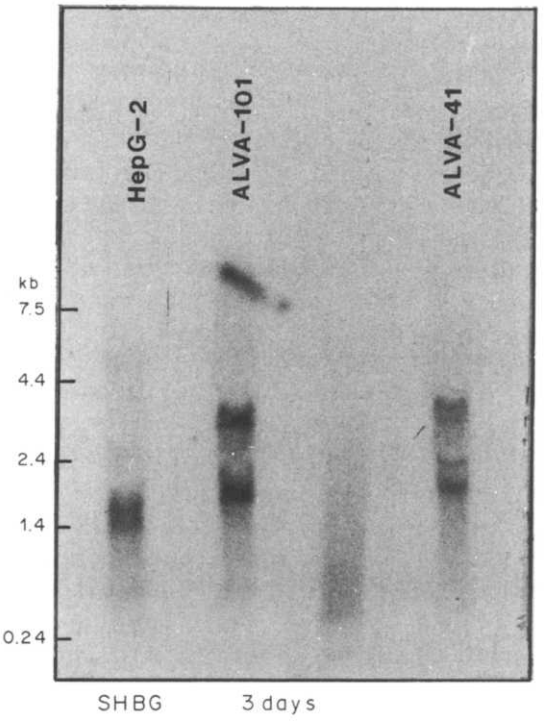

Fig. 7. Northern analysis of total cytoplasmic RNA from the human hepatoma cell line, HepG-2, ALVA-41 cells, and ALVA-101 cells. Hybridization was performed with a 550 bp cDNA for the 3′ portion of human SHBG.

DISCUSSION

The results of the studies presented in this paper suggest a possible dual role for SHBG in the regulation of prostatic carcinoma. The first of these is the classic action of this protein as an extracellular binding reservoir for testosterone. In this respect, the data presented demonstrates that SHBG can inhibit the entry of tumor cells. This function does not appear to be specific for SHBG, since albumin can also effectively inhibit the passage of DHT into the tumors *in vitro*. These data would then suggest that for this classic function of a binding protein, either albumin or SHBG can serve equally well. However, recent data *in vivo* has suggested that in some tissues, including the prostate, all of the testosterone that flows through the tissues, including that bound to albumin and SHBG, may be taken up by the tissue [18]. In addition, Hammond *et al.* [19] have demonstrated that corticosteroid binding globulin (CBG) bound cortisol may be targeted towards specific inflamed tissues and released from the cell surface. Therefore, *in vivo*, the binding effects that we have demonstrated *in vitro* may be modified. However, even if the SHBG-bound steroids were transported into the cell, the amount entering would be relatively trivial to passive entry of free steroid [15]. Therefore, the evidence for the effect of SHBG on steroid transport into the cell would still be in favor of that of an extracellular binding protein. A further aspect of steroid uptake that may be important is that although total steroid uptake may not be different between the two ligands, the rate of tissue uptake is slower with SHBG present [20]. However, the importance of rate has not yet been defined.

The second function of SHBG in relation to the prostate is that possible role as a growth factor (Loop S., paper in preparation). In the study we have presented in this paper, SHBG stimulates DNA synthesis, as measured by [^{3}H]thymidine uptake, in at least two prostate cancer cell lines. In addition, this phenomenon appears to be augmented by steroids in the ALVA-101 cells. All of the cell lines studied thus far have demonstrated high affinity receptors for the binding protein. However, since the SHBG preparation was prepared from human pregnancy serum, the possibility of a minor contaminating protein causing the effects that are seen on growth cannot be entirely ruled out. This possibility would be ruled out by the use of recombinant SHBG. Unfortunately, this material is not yet available.

Of further interest has been the demonstration of several transcripts in the prostate cell lines that hybridize to a cDNA for SHBG. One of these transcripts, 1.9 kb, is only slightly larger than the mRNA present in the liver which encodes for circulating SHBG. However, the second is significantly larger, 3.4 kb, and differs from the normal transcript at its 5′ end, as evidenced by its failure to hybridize with oligonucleotide to exon III. In this respect, the larger transcript may be analogous to the 2.9 kb testicular transcript described by Joseph [22]. In addition, SHBG is homologous to protein-S and the amino terminal portion of SHBG terminates where an epidermal growth factor (EGF) sequence begins on protein-S [23]. Therefore, if the larger transcript encodes for an SHBG-like peptide that is extended at its 5′ end, then this new peptide may well have EGF-like activity. If this hypothesis proves correct, as with other growth factors, e.g. insulin and insulin-like growth factor 1 (IGF-1), similar activity may be expressed through a single receptor for both molecules.

It should be noted that several mRNA species have also been recently reported in RNA isolated from the LnCAP human carcinoma cell line [21]. In addition, the origin of these multiple mRNA species may in part be explained by the location of the SHBG gene on the short aim of chromosome 17 adjacent to the P-53 tumor suppression gene. Since this is a region rich in adenosine and thymidine nucleotides, it would be prone to recombinant events. Therefore, a mutation of the P-53 gene associated with a recombinant event could explain the association between multiple mRNA species for the SHBG message and the development of a cancer due to the loss of an effective P-53 tumor suppressor gene [24, 25].

The expression of specific membrane receptors, the demonstration of specific mRNA species, and the demonstration of SHBG immunoreactivity in the cells clearly indicate an autocrine role for SHBG in human prostate tumor cells. The production of SHBG by a steroid dependent tumor cell line (ALVA-101) has multiple implications for the pathological advancement of the disease. It may provide an alternative mechanism for steroid recruitment or concentration of steroid in the tumor following androgen deprivation therapy. The direct stimulation of cellular proliferation by SHBG and the augmentation of this response by very low levels of androgen may further provide alternative mechanisms for androgen independent growth similar to that seen in androgen independent clones of the LnCAP cell line. For instance, the LnCAP-r subclone does not require androgens for growth, but the growth rate is stimulated by the addition of steroids [26].

The presence of an SHBG message, as well as SHBG immunoreactivity in the cells, suggests that the tumors may have the ability to produce their own growth factors [27]. The characteristic of malignant cells having autocrine activity with respect to growth factors has been demonstrated in the prostate for EGF and other putative growth factors. The production of SHBG by a steroid dependent tumor could also have the additional effect of providing a concentration mechanism for steroid in the cell, although this effect has not been demonstrated.

The importance of these activities of SHBG in the normal prostate has not been explored. However, in a group of men without clinical evidence of prostate cancer, a significant positive correlation was noted between serum levels of SHBG and PSA (unpublished data). In this same group of men no correlation was noted between serum testosterone or free testosterone and PSA. Also, in a single experiment, we did not find any hybridization of the SHBG cDNA to RNA from normal human prostate. These findings suggest that in the nonmalignant prostate, SHBG may have some stimulatory effect as evidence by the correlation with PSA, but unlike in malignant cells, this is an endocrine rather than an autocrine activity.

The concept of a serum transport protein having activity in addition to that of a ligand binding function is not unusual. For example, transferrin and IGF-I binding protein-I (IGF-BP-I) have been demonstrated to have growth factor-like activity [28, 29]. In the case of transferrin, this activity is through cell surface receptors for the protein, while in the instance of IGF-BP-I, this activity seems to be expressed through the IGF-I receptor. Therefore, it would now appear that we can add SHBG to this growing list of transport proteins with dual functions.

In summary, in the studies presented in this paper, we have demonstrated that SHBG or a related peptide can have several effects on the prostate: (1) It may act as a classic extracellular binding protein to exclude steroids from the cell, and (2) it appears to have a direct stimulatory activity on DNA synthesis in human prostate tumors through specific cell surface receptors.

In addition to these actions, SHBG or an SHBG-like peptide may be produced by malignant human prostate cells, and therefore may act as an autocrine growth factor.

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