BEYOND CARRIER PROTEINS

Sex hormone-binding globulin is synthesized in target cells

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Abstract

Sex hormone-binding globulin (SHBG) is a multifunctional protein that acts in humans to regulate the response to steroids at several junctures. It was originally described as a hepatically secreted protein that is the major binding protein for sex steroids in plasma, thereby regulating the availability of free steroids to hormone-responsive tissues. SHBG also functions as part of a novel steroid-signaling system that is independent of the classical intracellular steroid receptors. Unlike the intracellular steroid receptors that are ligand-activated transcription factors, SHBG mediates androgen and estrogen signaling at the cell membrane by way of cAMP. We have reviewed the current state of knowledge on the SHBG gene and the role of SHBG in steroid signaling (we shall not address its function as a plasma-binding protein).

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The sex hormone-binding globulin (SHBG) gene

The SHBG gene (Fig. 1) is located on chromosome 17p13.1, only 30 kb away from the p53 tumor-suppressor gene, and within a region known to undergo allelic deletions and mutations in a large variety of tumors (Cousin et al. 2000). Its proximity to p53 raises the unaddressed question of whether genomic events that alter the SHBG locus might also lead to changes arising in hormone-dependent cancers, e.g. breast and prostate. This question arises because, as we shall review, it is clear that SHBG is synthesized in these two tissues. Two major SHBG transcripts are known, each originating from a different promoter (minor SHBG transcripts have received little attention and will not be discussed here) (Gershagen et al. 1987, 1989, 1991, Hammond et al. 1987, 1989, Joseph et al. 1991, Bocchinfuso et al. 1992, Bocchinfuso & Hammond 1994, Hammond & Bocchinfuso 1996, Janne et al. 1998). The first major transcript encodes a precursor for the secreted (plasma) form of SHBG, and was originally described in the liver (SHBGt) (Que & Petra 1987), while the second encodes a protein of unknown function and was originally described in the testis (SHBGt) (Hammond et al. 1989).

SHBGt

SHBGt is encoded by eight exons, ranging in size from 90 to 208 bp. With the exception of a 733 bp intron separating exons 6 and 7 (which perhaps contains alternative splicing regulatory elements), the remaining introns are relatively small (133–331 bp). SHBGt is under the transcriptional control of a TATA-less promoter which possesses multiple protein-binding sites, including those for hepatocyte nuclear factor-4 and SP-1 (Janne & Hammond 1998, Hogeveen et al. 2001). The nascent SHBGt transcript encodes a precursor protein with a 29 amino acid, lysine-rich signal peptide (encoded within exon 1 and part of exon 2) at its amino terminus. The mature, secreted form of SHBG in human plasma lacks this signal peptide and circulates as a glycosylated, 92·5 kd homodimer (Khan et al. 1985, Hammond et al. 1986, Englebienne et al. 1987, Danzo et al. 1989, Grishkovskaya et al. 2000) containing two steroid-binding sites (Avvakumov et al. 2001).

SHBGt

The second major transcript, SHBGt, is regulated by an uncharacterized promoter that lies upstream of the SHBGt promoter (Hammond & Bocchinfuso 1996). SHBGt and SHBGt differ in their 5′ sequences and in the absence of exon 7 in SHBGt. The complete 5′ end sequence of SHBGt has not been reported; the incomplete sequence contains an initial, long open reading frame wherein the first ATG start codon does not appear until the shared
Local synthesis of SHBG

Figure 1 Structure of the human SHBG gene. The SHBG gene, and its position on chromosome 17p13.1, as set out in the December 2001 UCSC Human Genome Project Working Draft (URL: http://genome.ucsc.edu/cgi-bin/hgTracks?position=chr17:8117595-8120775&hgslid=7346710). The exon–intron structure of the two major SHBG gene transcripts, SHBG-L and SHBG-T, are shown, with exons represented by shaded boxes, and introns by lines with directional arrows. SHBG-L, the transcript for the secreted form of SHBG, originally described in the liver, consists of eight coding exons spanning just over 3 kb. The full sequence of SHBG-T, the transcript of unknown function originally described in the testis, is currently incomplete at its 5’ end. It shares sequences beginning with exon 2 of SHBG-L, but lacks exon 7. The SHBG gene lies only 30 kb away from the p53 tumor-suppressor gene. FISH, fluorescence in situ hybridization.

exon 2. Based on current information, SHBG\textsubscript{T} would encode a truncated version of the secreted SHBG precursor with a different carboxyl terminus. This protein would probably be unstable, as similar 5’ end truncations of SHBG\textsubscript{L} code for unstable proteins (Hildebrand \textit{et al.} 1995). If stable, the SHBG\textsubscript{T} protein would most likely not bind steroids, although it would possess the domain known to contain the site of SHBG that binds to its receptor (R\textsubscript{SHBG}) (Gershagen \textit{et al.} 1989, Joseph \textit{et al.} 1996, Kahn \textit{et al.} 1990).

SHBG-mediated steroid signaling through the SHBG receptor

The current view of SHBG function differs dramatically from the way in which it was originally conceptualized, e.g. to regulate the concentration of certain free steroids in plasma. Although of undeniable importance, this original model has been substantially broadened by the realization that SHBG is also part of a signal transduction system for steroids at the cell membrane.

The SHBG receptor

An active role for SHBG in steroid signaling was suggested initially by the discovery of specific, high-affinity binding sites for SHBG on uterine endometrial cell membranes (Strel’chyonok \textit{et al.} 1984), isolated prostatic cell membranes (Hryb \textit{et al.} 1985) and human placenta (Avvakumov \textit{et al.} 1985). Subsequently, SHBG binding was also demonstrated in MCF-7 breast cancer cells (Frairia \textit{et al.} 1991, Porto \textit{et al.} 1992\textsubscript{a,b}, Fissore \textit{et al.} 1994), normal breast (Frairia \textit{et al.} 1991, Fortunati \textit{et al.} 1992\textsubscript{a}) and epididymis (Guéant \textit{et al.} 1991, Felden \textit{et al.} 1992, Porto \textit{et al.} 1992\textsubscript{a,b}, Krupenko \textit{et al.} 1994), but not with striated muscle, colonic epithelia, or lymphocytes (Avvakumov \textit{et al.} 1985, Felden \textit{et al.} 1992, Fortunati \textit{et al.} 1992\textsubscript{a,b}, Frairia \textit{et al.} 1991, Porto \textit{et al.} 1992\textsubscript{a,b}, Krupenko \textit{et al.} 1994). The binding properties of SHBG are consistent with the presence of a specific R\textsubscript{SHBG} on cell membranes, and the biochemistry of SHBG–R\textsubscript{SHBG} binding is well characterized. Foremost, R\textsubscript{SHBG} only binds steroid-free SHBG. All steroids that bind to SHBG inhibit the binding of SHBG to R\textsubscript{SHBG}; the magnitude of the inhibition is directly proportional to the magnitude of the association constant for the steroid–SHBG interaction (Fig. 2) (Hryb \textit{et al.} 1989, 1990). Once bound to R\textsubscript{SHBG}, SHBG binds steroids with affinities equal to SHBG that is in solution (Hryb \textit{et al.} 1990). The SHBG domain, or at least a portion of it, that interacts with R\textsubscript{SHBG} has been localized to a ten amino acid stretch (TWDPEGVIFY) (Khan \textit{et al.} 1990) encoded within exon 3. This region is shared between SHBG\textsubscript{L} and SHBG\textsubscript{T}, and is the most highly conserved portion of the molecule, both across species (Khan \textit{et al.} 1990) and in related proteins, e.g. protein S, laminin A, merosin, and \textit{Drosophila crumbs} protein (Gershagen \textit{et al.} 1987, Khan \textit{et al.} 1990, Joseph & Baker 1992). Although there is a substantial body of knowledge about R\textsubscript{SHBG}, its structure remains elusive; the R\textsubscript{SHBG} gene has yet to be identified and characterized.

Steroid activation of cAMP through R\textsubscript{SHBG}

Our current conception of SHBG–R\textsubscript{SHBG}–steroid signaling is shown in Fig. 3. As discussed above, a specific sequence of events is necessary to initiate signaling through R\textsubscript{SHBG}, binding of unoccupied SHBG to R\textsubscript{SHBG} on the cell membrane, followed by binding of steroid to the SHBG–R\textsubscript{SHBG} complex, thereby activating it. Activation of R\textsubscript{SHBG} induces the synthesis of cAMP which, in turn, triggers downstream signaling and initiates genomic effects through the activation of promoters containing cAMP responsive elements (Nakhla \textit{et al.} 1990, Rosner \textit{et al.} 1992). These events occur too rapidly to be affected either by the dissociation of SHBG–R\textsubscript{SHBG}, seen subsequent to binding of the agonist, or by the transcriptional activation of classical steroid hormone receptors.
Furthermore, inhibitors of the transcriptional activation of the estrogen receptor and androgen receptor (AR) do not affect the cAMP response, supporting the independence of this pathway.

$R_{\text{SHBG}}$ appears to be coupled to a G-protein. There is a dose-related decrease in the binding of SHBG to $R_{\text{SHBG}}$ after incubation of the receptor preparation with the non-hydrolyzable GTP analogue, guanylyl-5'-imidodiphosphate (Nakhla et al. 1999), a phenomenon typical of the behavior of receptors coupled to G-proteins. In addition, in COS-1 cells, which express a functional $R_{\text{SHBG}}$ expression of dominant negative mutants of the G-protein $\alpha$-subunit (Osawa & Johnson 1991), cause a decrease in $R_{\text{SHBG}}$-mediated cAMP signaling (Nakhla et al. 1999).

Steroids that bind to SHBG act as either agonists or antagonists of $R_{\text{SHBG}}$-mediated signaling. Furthermore, whether or not a steroid is an agonist of $R_{\text{SHBG}}$-mediated signaling appears to be dependent on cell type. In the prostate, two steroids, estradiol and 5α-androstan-3α,17β-diol (3α-diol) are potent agonists (Nakhla et al. 1990, 1995). In fact, 3α-diol, which is active in this system at physiologic concentrations, was previously thought to be an inactive metabolite of dihydrotestosterone (DHT). Other steroids that bind SHBG with high affinity, e.g. DHT, testosterone, and 2-methoxyestradiol, are not agonists, but instead antagonize the effects of 3α-diol. On the contrary, DHT is an agonist for SHBG-$R_{\text{SHBG}}$ in both the LNCaP prostate cancer cell line (Nakhla et al. 1990) and in cultured human placenta (Queipo et al. 1998). Not surprisingly, the degree to which agonists induce cAMP through $R_{\text{SHBG}}$ appears to vary with cell type. For instance, the fractional increase in cAMP in cultures of human (Nakhla et al. 1994) and canine prostate (Nakhla et al. 1995) far exceeds that seen in LNCaP cells. It should not be lost sight of that, in both LNCaP cells (Nakhla et al. 1990) and placenta (Queipo et al. 1998), SHBG in the absence of steroid causes a modest increase in cAMP. Although the relationship between steroidal structure and affinity for SHBG has been examined in some detail (Cunningham et al. 1979, 1981), those studies shed no light on whether a given steroid might be an agonist or antagonist in the SHBG-$R_{\text{SHBG}}$ system.

**Biologic effects of steroid signaling through $R_{\text{SHBG}}$**

**Induction of prostate specific antigen (PSA) in prostate cells**

Delineation of the biologic effects of SHBG signaling through $R_{\text{SHBG}}$ has lagged behind our understanding of the biochemical analysis of its signaling pathway. Details regarding the downstream effects of steroid signaling through SHBG exist, but are not extensive. A downstream event of potential biologic importance is the intersection of this pathway with an AR-mediated event, the activation of the PSA gene and secretion of its translational product (Nakhla et al. 1997). The human PSA gene possesses an androgen response element in its promoter, and is transcribed upon activation of the AR in prostate cells. Prostate explants secrete PSA when treated with DHT; however, they do not when treated with estradiol, which does not bind to the AR. When such explants were treated first with SHBG, and then with estradiol, they produced PSA at concentrations similar to those seen when they were exposed to DHT. Furthermore, inhibitors of estrogen receptor activation did not block estradiol–SHBG–$R_{\text{SHBG}}$-mediated PSA induction, whereas inhibitors of AR activation did. These results indicate that estradiol–SHBG–$R_{\text{SHBG}}$ initiates ligand-independent activation of PSA secretion.

**Cell growth**

$R_{\text{SHBG}}$ signaling affects growth in two different cell lines, with opposite results. It decreases the estrogen-mediated growth of the human breast carcinoma cell line, MCF-7.
Figure 3 The SHBG signaling system. In its steroid-free configuration, SHBG binds to $R_{\text{SHBG}}$ on cell membranes, forming a bipartite complex (SHBG–$R_{\text{SHBG}}$). SHBG, already bound to a steroid, non-competitively inhibits the binding of SHBG to $R_{\text{SHBG}}$. However, within minutes after exposure of SHBG–$R_{\text{SHBG}}$ to a steroid agonist, e.g. estradiol (Nakhla et al. 1990, 1994) or 5α-androstan,3α,17β-diol (Nakhla et al. 1995), a tripartite complex (steroid–SHBG–$R_{\text{SHBG}}$) forms that activates adenylyl cyclase, leading to the generation of the second messenger, cAMP.
Figure 4 SHBG expression in normal prostate (from Hryb et al. 2002) (A) In situ hybridization (×400). A 5µm human prostate section was processed using the Biogenex (San Ramon, CA, USA) super sensitive in situ hybridization kit. RNase activity was blocked and the section was incubated with a 521 bp human SHBG cDNA probe (prepared by PCR incorporation of biotin-14-dCTP). After heating and incubation, slides were developed using the ABC method (ABC elite system; Vector Labs, Burlingame, CA, USA), using DAB as the substrate, and counterstained with hematoxylin. Photographs were taken with a 35mm camera mounted to a BX60 microscope and digitized. (B) Immunohistochemistry (×400). A 5µm human prostate section was fixed and incubated overnight at 4°C with a rabbit anti-SHBG polyclonal antiserum (64–4), generated in our laboratory. The section was developed by the aviden–biotin complex (ABC) method using DAB as the substrate, and counterstained with hematoxylin. Photographs were taken as above.
undertook an examination of human prostate and breast tissue sections by in situ hybridization and immunocytochemistry. In the prostate, cells that expressed SHBG mRNA (Fig. 4A) also stained for SHBG protein with a monospecific, polyclonal rabbit anti-SHBG (64–4) (Fig. 4B) or monoclonal antibodies (data not shown). Comparable results were obtained for breast tissue (authors’ unpublished observations). While we cannot dismiss internalization of plasma SHBG as at least a partial source of the immunoreactive SHBG in these studies, it is likely that locally produced SHBG is the major species in these cells. If so, regulated SHBG synthesis and secretion in the breast and prostate could affect intracellular free steroid concentrations and participate in RS

HM induced cAMP elevation is solely responsible for these observations, or whether other factors involved in growth regulation play a role, remains to be investigated. Furthermore, these are cancer cell lines; whether signaling through RS

HM has the same effects on normal breast and prostate epithelial cells is not known. On a very speculative note, if this relationship exists in normal cells, SHBG might be considered a tumor-suppressor gene in breast cancer, and agonists of the SHBG–RS

HM Pathway might be used to suppress the malignant phenotype, while antagonists of SHBG–RS

HM signaling might be useful in prostate cancer, where inhibition is wanted.

Localized expression of SHBG in hormone-responsive tissues

The presence of SHBG in cells that respond to sex steroids has been examined in a number of laboratories. Early immunohistochemical studies, using rabbit polyclonal antisera, showed SHBG antigen in both the prostate and breast (Bordin & Petra 1980, Tardivel-Lacombe et al. 1984, Sinnecker et al. 1988, 1990, Meyer et al. 1994, Germain et al. 1997). However, whether SHBG was delivered to these cells through the plasma or was locally expressed remained a question. Indeed, although all the antisera were raised using highly purified SHBG, there was no proof that this intracellular antigenic activity was SHBG, rather than a related antigen.

More recently, SHBG mRNA has been demonstrated in a number of non-hepatic tissues and cell lines (Larrea et al. 1993, Misao et al. 1994, 1997, Moore et al. 1996, Murayama et al. 1999). Although the data in the cell lines that stain for SHBG protein, and show SHBG mRNA by RT-PCR and/or by Northern blotting, are convincing, the conclusions based on experiments using human tissue sections are ambiguous. With one exception (Noe 1999), studies showing the tissue mRNA did not show the protein, and those demonstrating the protein did not show the mRNA. In the one exception, Noe (1999) detected both the protein (immunostaining) and the mRNA by RT-PCR in human Fallopian tubes. However, no studies were presented to ascertain whether the mRNA was translated, e.g. the possibility remained that the mRNA was not translated and the protein arrived via the plasma. Although it is possible to demonstrate the causal relationship between an mRNA and its protein in cell lines, this cannot be done in tissue sections. The strongest inferential evidence that is possible, under these circumstances, is to show that the mRNA (in situ hybridization) and the protein exist in the same cells. Thus, we (Hryb et al. 2002)


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