Journal of Andrology, Vol. 23, No. 5, September/October 2002

Review

Androgen-Binding Protein and Reproduction: Where Do We Stand?

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Received for publication February 26, 2001; accepted for publication November 27, 2001.

Androgen-binding protein (ABP) is a testicular glycoprotein (French and Ritzén, 1973; Danzo et al, 1974; Danzo and Black, 1990) that binds androgens with high affinity (Westphal, 1986) and transports them to the epididymis (French and Ritzén, 1973). The first evidence of ABPs existence came from the early 1970s, when a protein with a steroid-binding activity similar to the androgen receptor was detected in rat testis (French and Ritzén, 1973; Danzo et al, 1974). Subsequent studies revealed that the protein was secreted by rat Sertoli cells (Fritz et al, 1974; Tindall et al, 1974) and was very similar to a plasma protein described a few years earlier that was produced by the liver and bound dihydrotestosterone (DHT), testosterone, and estradiol (Hammond et al, 1987). The plasma protein is referred to by a variety of names, including sex hormone-binding globulin (SHBG), sex steroid—binding protein (SBP), and testosterone—estradiol—binding globulin (Joseph, 1994). Further purification of both proteins by affinity chromatography (Musto et al, 1980), as well as their characterization by photoaffinity labeling (Danzo et al, 1980) and immunoassay (Cheng et al, 1984), confirmed that ABP and SHBG were very similar physicochemically. The determination of the complete amino acid sequence of human SHBG/ABP (Walsh et al, 1986) and the cloning of the rat ABP (Joseph et al, 1985; Reventós et al, 1986) and human SHBG/ABP (Hammond et al, 1987) complementary DNAs (cDNAs) proved that both proteins share the same primary amino acid sequence, even though they differ in their carbohydrate content (Danzo and Bell, 1988). Lastly, results from Southern blot analysis of genomic DNA revealed that only 1 gene exists for SHBG/ABP in the human genome (Hammond et al, 1989).

During the past 25 years, numerous efforts have been made to define possible roles for SHBG/ABP in spermatogenesis, but its fundamental action is still obscure. What is clear is that the SHBG gene encodes different messenger RNAs (mRNAs), each with possible dissimilar functions. In the present

review, we summarize what is known about this protein and the questions that are still unsolved, and we discuss the potential roles of SHBG/ABP in spermatogenesis.

Structural Data: From Gene to Protein

The appropriate use of the terms "SHBG" and "ABP" is still a matter of significant debate. Joseph (1994) proposed to name the plasma form SHBG and the testicular form ABP. However, given that SHBG can be detected in tissues other than the testis and liver, the existence of more than 1 form of SHBG, the putative presence of the plasma protein in the human testis, and the putative binding of SHBG to other steroids besides androgens, we propose that an unified nomenclature should be used for all the forms of SHBG and ABP. In the present review, we will use the term "SHBG/ABP" independently of the source of its expression.

The SHBG gene has been localized to 17p13-p12 of the human genome (Berube et al, 1990) and in regions of the 11th and 10th chromosomes of the mouse and rat, respectively, that share considerable homology with the human SHBG/ABP region (Joseph et al, 1991a; Sullivan et al, 1991).

Although the cDNAs for SHBG/ABP have been cloned and sequenced from many different species, the public databases only contain the complete genomic sequence of the human, rat, and rabbit. The structural organization of the 3 genes is highly conserved. The coding regions of human, rat, and rabbit SHBG/ABP span 3.2, 3, and 2.5 kb, respectively, and consist of 8 exons separated by small introns. Consensus splice sites are present at all exon—intron junctions. Repetitive elements have been found in the human and rat genes but not in the rabbit SHBG/ABP. Specifically, within the human gene, repetitive *A*/*u* sequences are present in the 5'-flanking region and in intron 6 (Hammond et al, 1989; Ip et al, 2000), regions suggested to have important regulatory functions (Joseph, 1994).

The 5'-flanking regions of human, rat, and rabbit SHBG/ABP do not show a regular DNA sequence TATA box, a DNA sequence CAAT box, or an initiator sequence (Ip et al, 2000). However, the detection of high levels of rat SHBG/ABP mRNA expression in the testis of the transgenic mice carrying the rat SHBG/ABP gene containing exclusively 1.5 kb of the promoter confirms the presence of a transcription start site within this part of the sequence (Reventós et al, 1993). A recent study using the mouse Sertoli cell line MSC-1 reported the presence of the major transcription start site at 36 bp upstream of the initiating methionine (Met) residue and a minor start site at 179 bp upstream of the major site. Neither mutations of the putative modified TATA sequence nor modifications of a consensus RNA splice sequence at the start site vary the activity of the promoter (Fenstermacher and Joseph, 1998).

The gene expression studies performed in the last decade revealed a more complex structure of the SHBG gene. Alternate exon 1 sequences have been identified in the human and rat genomes. These sequences are located 1.9 kb upstream of the previously described initiation codon in the human SHBG gene (Hammond et al, 1989) and 15 kb upstream in the rat SHBG/ABP gene (Joseph, 1994). The corresponding alternate promoters have been localized adjacent to these alternate exons.

The transcription of the SHBG/ABP gene results in different mRNAs. In the rat, the first mRNA characterized was the 1.7-kb form that contains exons 1 to 8 and encodes a 403-amino acid residue precursor of 44 539 daltons. The amino terminus of this precursor protein contains a signal peptide that is removed and results in a mature protein of 373 residues and 41 183 daltons (Joseph, 1994). This protein contains 4 cysteine residues that form 2 disulfide bridges in the mature protein and 2 potential sites of asparagine (Asn) glycosylation (Joseph, 1994) and correspond to the secreted protein found in plasma. The human SHBG/ABP-secreted protein is also encoded by exons 1 to 8. The length of the resultant transcript is 1.6 kb. The human signal peptide sequence is very similar but

is slightly smaller than the one found in the rat SHBG/ABP sequence. The mature human protein is a polypeptide of 373 residues that shares 68% residue identity with the rat SHBG/ABP (Hammond et al, 1989). The cysteine residues are conserved in the human protein, and there are 3 sites of glycosylation: 2 of Asn at the carboxyl terminus and 1 of O-glycosylation at the amino-terminal region.

The existence of alternate SHBG/ABP mRNA species has been demonstrated in human and rat tissues. Specifically, 1 of the 3 cDNAs isolated in the human testis is identical to the human liver cDNA, but its 5' end is 116 bp shorter. The other 2 testicular sequences contain alternative first exons followed by exons 2 to 8, but a 208-bp region, corresponding to exon 7, is missing in one of them (Hammond et al, 1989) (Figure 1).

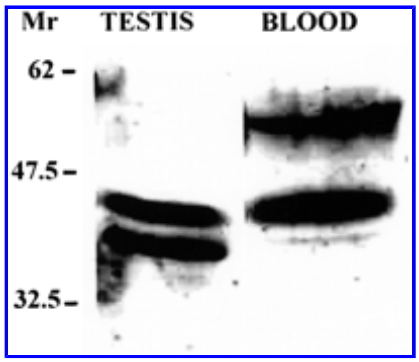

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Figure 1. Western Blot analysis of sex hormone-binding globulin/androgen-binding protein (SHBG/ABP) in human testis. Lane 1 represents total protein extracted from a human testis of an organ donor, and lane 2 is of diluted serum from a healthy volunteer. Specific SHBG bands are labeled with arrows. Two bands sized 52 and 48 kd were detected in the testicular extract that could correspond to the differentially glycosylated forms described in serum.

In rats, alternate SHBG/ABP cDNAs have been identified in the testis, liver, and brain (Joseph et al, 1991b; Joseph, 1994). One cDNA contains an alternate exon 1, followed by exons 2 to 8, and the other has the same sequence but is lacking exon 6 (Sullivan et al, 1993). The resultant protein has a modified N-terminal sequence and does not appear to have a signal peptide. The expression of the recombinant alternate (Alt)-ABP/SHBG in the COS cell line showed that the alternate protein was not secreted, and it was not known if it bound steroids (Sullivan et al, 1993). In subsequent studies, nuclear localization of the Alt-SHBG/ABP was demonstrated, indicating that the alternate N-terminal sequence targets Alt-ABP/SHBG to the nucleus instead of the endoplasmic reticulum (Joseph et al, 1996). The region that interacts with the plasma membrane receptor was present in all the forms, suggesting that, although the role of these alternate testicular proteins is probably different from the function of the secreted SHBG/ABP, the plasma membrane interaction may be a common mechanism of action for all the described forms (Sullivan et al, 1993).

Functional Data: Characteristics of the Protein

The SHBG/ABP protein exists as homodimers in biological fluids. In each monomer, 3 functional domains have been identified: a steroid-binding domain, a dimerization domain, and a plasma membrane receptor-interacting domain (Joseph et al, 1994).

The affinity of the SHBG/ABP protein for DHT (K_a 1 x 10^9 to 1 x 10^{10}) is very high, slightly lower (1 order of magnitude) for testosterone and estradiol, and very low for other biologically active steroids. These data imply that the SHBG/ABP and androgen receptor affinity for DHT are similar. The

half-life of the DHT-SHBG/ABP complex is 30 to 60 minutes for the human protein and 5-6 minutes for the rat protein (Westphal, 1986; Joseph, 1994). Different approaches have been used in an attempt to localize the steroid-binding domain in several regions of the rat protein (Joseph, 1994). By using site-directed mutagenesis, it was demonstrated that nonconservative modifications throughout the complete rat SHBG/ABP primary sequence changed the steroid-binding and secretion properties of the protein (Joseph and Lawrence, 1993). By photoaffinity labeling, Danzo et al (1991) identified the steroid-binding region in residues 141 to 150 of the rat protein. In the human protein, by combining affinity labeling and recombinant mutant experiments, it was demonstrated that the region adjacent to Met-139 was important for steroid binding (Grenot et al, 1992; Bocchinfuso and Hammond, 1994). Moreover, the expression of several recombinant human SHBG/ABP deletion mutants in *Escherichia coli* demonstrated that residues 18 to 177 of the human protein contained all the essential elements for the steroid binding and dimerization and that these 2 processes were probably interdependent (Hildebrand et al, 1995).

The likely existence of a membrane receptor-binding domain for SHBG/ABP was supported by experiments in which its interaction with proteins of the plasma membranes of several human target tissues was documented. Binding of SHBG/ABP to prostate, testis, decidual endometrium, breast tissue and breast cancer cells, and liver (but not to muscle or colon) was demonstrated, leading the authors to suggest that the SHBG/ABP receptors are located only in sex steroid—dependent tissues that contain estrogen receptors (Frairia et al, 1992). A region important for this receptor-binding activity was localized to residues 48 to 57 of the human protein by comparing the binding affinity of the purified human SHBG/ABP with digested fragments of the protein for the prostate receptor (Khan et al, 1990). This short sequence is identical in rats and humans and is part of a region highly conserved between both species. Several models for the binding of SHBG/ABP to membranes have been proposed: the prostate model states that only unliganded SHBG/ABP could bind to the receptor and that the complex formed by SHBG/ABP receptor will allow the binding of the steroid (Hryb et al, 1990). The membrane receptor described in testicular tissue presents similar characteristics to the prostate receptor (Porto et al, 1992). In endometrium, the membrane receptor was described to interact with SHBG/ABP—estrogen complexes as well as unliganded SHBG/ABP, but not with androgen-bound SHBG/ABP (Fortunati et al, 1992). Recently, a function of the SHBG/ABP membrane receptor serving as a negative regulator of cellular growth in breast and prostate cancer was reported. It has also been demonstrated that SHBG/ABP inhibits the estradiol-induced proliferation by inducing cyclic adenosine monophosphate (cAMP) and activating protein kinase A (PKA) (Fortunati et al, 1996; Nakhla et al, 1997) and that estradiol led to a significant increase in cAMP only in the presence of SHBG/ABP. The authors conclude that the ability of the estradiol to induce cAMP is mediated, in part, by SHBG/ABP (Fortunati et al, 1999).

The comparison of the SHBG/ABP sequence with the Protein Data Bank allowed the identification of a tandem repeat of 2 laminin G-like domains. The 2 G domains constitute a unit called the SHBG-like domain that is present in the ligands of the Tyro-3 receptor protein—tyrosine kinase family such as the growth arrest-specific protein 6 (GAS 6) and protein S as well as in the extracellular matrix protein laminin α-chain (Joseph and Baker, 1992). Recently, the high-resolution crystal structure of the N-terminal G domain of human SHBG/ABP complexed with 5α-DHT was solved, confirming the dimeric conformation of the protein, the existence of 2 separate steroid-binding pockets, the calcium-binding sites per dimer, and the intercalation of the steroid between 2 ß-sheets of a jellyroll structure (Grishkovskaya et al, 2000). The structure of the steroid-binding pocket is in concordance with mutagenesis and photolabeling experimental results demonstrating that residues in contact with the steroid are highly conserved in all the species studied. The steroid-binding sites do not participate in the dimerization interface and, although dimer formation may occur in the absence of calcium, this divalent cation can promote dimer formation. With respect to the membrane receptor,

only 5 (48-52) of the 9 residues identified by peptide mapping are partially exposed on the surface, but an alternative receptor-binding site has been proposed in the immediate vicinity of the steroid-binding pocket that could explain the steroid dependency of the interaction (Grishkovskaya et al, 2000).

Source of SHBG/ABP Expression

The presence of SHBG/ABP has been identified in numerous species. In humans, the major sources of SHBG/ABP are the liver (Khan et al, 1981) and the testis (Hammond et al, 1989; Wang et al, 1989). Testicular SHBG/ABP has been found in all mammalian species examined (human, monkey, rat, mouse, rabbit, lamb, and ram) as well as in distantly related species such as teleost fishes and sharks (Joseph, 1994). However, while in humans, the SHBG/ABP is synthesized in the liver and testis during both fetal and adult life, the liver of rodents expresses SHBG/ABP only during the fetal period (Carreau, 1986).

Within the rodent testis, there is evidence that the SHBG/ABP is synthesized and secreted by Sertoli cells (Hagenas et al, 1975; Oke and Suárez-Quian, 1993; Joseph, 1994). Although it has been suggested that rat germ cells could also synthesize the protein, no convincing data exist to support this hypothesis. In contrast, only limited studies are published regarding the origin of human testicular SHBG/ABP. Two reports demonstrated the presence of the protein inside the cytoplasm of Sertoli cells (Forti et al, 1989; Gerard et al, 1996), and an additional publication showed the secretion of SHBG/ABP by Sertoli cells in culture and its regulation by follicle-stimulating hormones (FSHs) (Santiemma et al, 1992).

Transgenic mice overexpressing rat and human SHBG/ABP illustrate the different localization of SHBG/ABP expression in each species. Transgenic male mice carrying a 5.5-kb genomic DNA fragment containing the entire coding regions and 1.5 kb upstream of the transcription start site of rat SHBG/ABP express high amounts of SHBG/ABP in the adult testis but not in the liver (Reventós et al, 1993), and the cellular type responsible for the testicular expression is the Sertoli cell (Esteban et al, 1997a; Joseph et al, 1997a). In contrast, in transgenic mice overexpressing the human gene, the human SHBG/ABP transcripts were expressed abundantly in the liver and testis (Jänne et al, 1998). Several lines of mice carrying the human SHBG/ABP gene have been produced containing either an 11- or a 4.4-kb genomic fragment (Jänne et al, 1998). Interestingly, the testicular expression was much lower in the transgenic mouse lines that contain the 4.3-kb fragment and do not express the alternative form than in the lines containing the longer genomic fragment (11 kb). The majority of testicular transcripts in the 11-kb transgenic lines corresponded to the alternative form. The predominance of the P1 transcripts in the testis of the 4.3-kb human SHBG transgenic lines (Jänne et al, 1998) and the rat SHBG transgenic line (Selva et al, 2000) could be due to the absence of the alternative promoter in the transgene. However, the lower abundance of the P1 transcripts in the testis of the 4.3-kb human SHBG transgenic lines compared to the rat SHBG/ABP transgenic mouse line, together with data demonstrating the predominance of the P1 promoter transcripts over the PA promoter transcripts in the normal rat testis (Sullivan et al, 1993), suggests a higher transcriptional activity of the P1 promoter in rat testis, whereas in human testis, the PA promoter is predominant. Supporting this hypothesis, the SHBG transcripts identified in the human testis do not contain exon 1 sequences (Gershagen et al, 1989; Hammond et al, 1989).

In recent years, the expression of SHBG/ABP was described in many other tissues. In the female reproductive tract, SHBG/ABP expression was detected in the ovary, oviduct, and uterus of transgenic female mice overexpressing rat SHBG/ABP (Joseph et al, 1997b). In humans, SHBG/ABP variants were demonstrated in ovarian endometriosis (Misao et al, 1998b) and uterine endometrium (Misao et al, 1997a). In the nervous system, secreted and alternate forms of SHBG/ABP were found in several

regions of the fetal and adult rat brain (Wang et al, 1990; Becchis et al, 1996) and also in the human fetal brain (Benavides et al, unpublished data). Transgenic mice overexpressing the human SHBG/ABP gene revealed the presence of the SHBG/ABP mRNA in the kidney and the developing duodenum (Jänne et al, 1998, 1999). Moreover, SHBG/ABP expression was found in several types of hormonally regulated human neoplasias, such as carcinoma of the breast (Moore et al, 1996; Murayama et al, 1999), ovary (Misao et al, 1998a), endometrium (Misao et al, 1997c), cervix (Misao et al, 1997b), and prostate (Mercier-Bodard et al, 1991; Plymate et al, 1991; Selva et al, unpublished data).

Hormonal Regulation of SHBG/ABP Expression

Several hormones including FSH, androgens, estrogens, GH, insulin, and prolactin are reported to regulate the expression and/or secretion of SHBG/ABP, although this is an area that remains open to considerable debate. Whether the response is positive or negative for a given stimuli, as well as the intensity of the response to the stimuli, for example, depends on several factors: the age of animals, the dose of hormone, the cellular type (eg, hepatocyte or Sertoli cell), and, of course, the presence of other paracrine or endocrine factors (whole-animal models vs isolated cells in culture) in which the response has been determined to have contributed to this current state of confusion regarding the hormonal regulation of SHBG/ABP regulation.

SHBG/ABP diminished after hypophysectomy but reappeared after FSH administration (Hansson et al, 1973; Weddington et al, 1975; Elkington et al, 1977). The upregulation of the testicular SHBG/ABP mRNA by FSH and testosterone was also demonstrated in 37-day-old hypophysectomized rats (Reventós et al, 1988). Using chemically hypophysectomized rats treated with a gonadotropin-releasing hormone (GnRH) antagonist combined with FSH, human chorionic gonadotropin (hCG), testosterone, or estradiol, Danzo et al (1990) demonstrated that FSH played a minor role in the SHBG/ABP production. Their results indicate that the primary in vivo regulator was testosterone, because testosterone and hCG were the most potent stimulators of SHBG/ABP production by the testis of 20- and 30-day-old rats, even in the presence of the GnRH antagonist. Interestingly, early studies demonstrated that testosterone alone was sufficient to maintain SHBG/ABP secretion, but only if it was administered immediately after hypophysectomy, prior to seminiferous tubule regression (Hansson et al, 1976). Further, the testosterone treatment produced an increase in SHBG/ABP at high doses but an SHBG/ABP reduction at low doses (Weddington et al, 1976).

The development of primary Sertoli cell cultures allowed investigators to test directly the ability of FSH and testosterone to regulate SHBG/ABP production and secretion. Whereas in culture, Sertoli cell SHBG/ABP secretion depends on the presence of FSH (Fritz et al, 1976; Skinner et al, 1989; Hall et al, 1990), analysis of SHBG/ABP mRNA levels after FSH and testosterone treatment led to conflicting results: induction of SHBG/ABP mRNA occurred in Sertoli cell primary cultures treated with FSH and testosterone (Reventós et al, 1986), but FSH was reported unable to induce the 1.7-kb SHBG/ABP transcript, instead maintaining its steady-state level and increasing an alternative 2.3-kb SHBG/ABP mRNA form (Hall et al, 1990). Further, it was suggested that the hormonal regulation of SHBG/ABP was indirect, not mediated by a cAMP response element (Joseph, 1994) but possibly mediated by androgen-regulated paracrine factors (Skinner and Fritz, 1985). A recent analysis of the SHBG/ABP P1 promoter using the mouse Sertoli cell line MSC-1 cotransfected with a DNA sequence (pCMVAR), which encodes the androgen receptor, showed that neither androgens nor FSHs were able to directly regulate the gene in this model system (Fenstermacher and Joseph, 1998). Since it is known that the rat SHBG/ABP gene contains 2 androgen regulatory half-site elements (Joseph et al, 1994), additional experiments will be necessary to resolve the issue of its direct regulation by FSHs and androgens.

The expression of SHBG/ABP mRNA is not constant throughout all the stages of the seminiferous epithelium. Using transillumination combined with microdissection of the seminiferous tubules into

the cyclic stages, the highest levels of SHBG/ABP mRNA were found in stages VII and VIII, but the SHBG/ABP protein did not appear until later, in stages IX and X (Ritzén et al, 1982). Androgen receptor mRNA showed a similar distribution, although the highest values were found in later stages (Isomaa et al, 1985). Recently, we have analyzed the SHBG/ABP and androgen receptor mRNA expression at specific stages of spermatogenesis in rat seminiferous tubules harvested by Laser Capture Microdissection. In contrast to the previously published data, we found the highest expression of ABP/SHBG mRNA in stages III and XII, whereas no expression was detected in stages VII and VIII. The expression of androgen receptor mRNA was found to be high in stages VII and III and low in XII (in preparation).

The ability of glucocorticoids to transcriptionally regulate the SHBG/ABP expression was demonstrated in rat Sertoli cell cultures by the increase in SHBP/ABP mRNA after dexamethasone administration and its blockade by actinomycin-D and not by cycloheximide pretreatment (Lim et al, 1996).

In hepatocyte cell cultures, estrogens and androgens increased SHBG/ABP levels (Lee et al, 1987), whereas insulin and prolactin inhibited its production (Plymate et al, 1988). In the testis, although the synthesis of estradiol by P450 aromatase was described many years ago (van der Molen et al, 1981; Papadopoulos et al, 1986; Carreau et al, 1988), the importance of this sex steroid in the maintenance of spermatogenesis was demonstrated only recently (Lubahn et al, 1993; Robertson et al, 1999; Pentikäinen et al, 2000). The demonstration of aromatase activity in Sertoli cells raises the possibility that estrogens could also serve to regulate the production of SHBG/ABP. In this regard, estrogens might be the effectors of some indirect actions of androgens. For example, in the testes of testicular feminized rats lacking a functional androgen receptor (Hansson et al, 1976), it should be interesting to explore the role of estrogens in maintaining the levels of SHBG/ABP.

Role of SHBG/ABP in Reproduction

Testicular SHBG/ABP protein was the first Sertoli cell secretory product identified with certainty and the first protein marker for Sertoli cell function and development. The role of SHBG/ABP in spermatogenesis has long been associated with the regulation of steroid levels in the testis and epididymis (French and Ritzén, 1973). Many of the observations made during the past 25 years have been consistent with this function, but the cell types where this regulation occurs are still a matter of debate. The rat Sertoli cell secretion of SHBG/ABP, for example, is bidirectional, 80% directed toward the seminiferous tubular lumen and 20% secreted basally, where it enters the blood (Gunsalus et al, 1980). Preliminary reports describing SHBG/ABP immunostaining in random rat testicular sections suggested a differential staining pattern (apical or basal) as a function of the cycle of the seminiferous epithelium (Pelliniemi et al, 1981). In contrast, using improved immunohistochemical protocols and a rabbit antiserum directed against rat androgen-binding protein, SHBG/ABP immunostaining was observed to span the complete height of the Sertoli cell in all stages of the cycle (Figure 2) (Oke and Suárez-Quian, 1993).

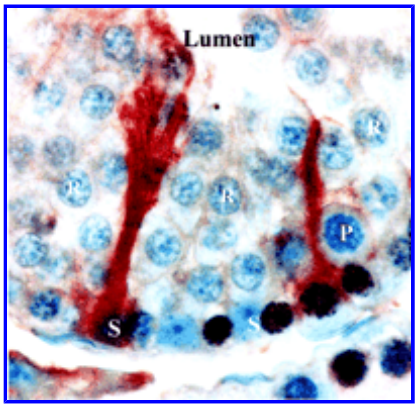

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Figure 2. Sex hormone-binding globulin/androgen-binding protein (SHBG/ABP) immunolocalization in rat Sertoli cells. The positive reaction product, here seen in red, extends the height of the Sertoli cell from the base of the seminiferous tubule to the lumen.

After secretion into the seminiferous tubule lumen, SHBG/ABP is transported to the epididymis along with mature spermatozoa. Its presence has been identified by immunohistochemistry in the Golgi region of the principal epithelial cells of the caput epididymis (Feldman et al, 1981; Pelliniemi et al, 1981) and in coated structures, endosomes, multivesicular bodies, and the Golgi apparatus of these cells by autoradiographic analysis (Gerard et al, 1988) and immunohistochemistry (Figure 3). More recently, in a rigorous light and ultrastructural immunohistochemistry study, region-specific SHBG/ABP endocytosis in the epididymis (principal cells of the initial segment and the intermediate zone) was observed, but additionally, SHBG/ABP immunostaining was detected in secretory vesicles of principal cells, an observation that led the authors to postulate the possibility of an epididymal-secreted form of SHBG/ABP (Hermo et al, 1998). However, since the expression of SHBG/ABP mRNA has still not been demonstrated in the epididymis, the secreted form of the epididymal principal cells could correspond to recycled SHBG/ABP, initially taken up by these cells, and not de novo synthesis. Similarly, endocytosis of SHBG/ABP by coated vesicles also occurs in male germ cells of monkeys (Gerard et al, 1991) and rats (Gerard et al, 1994), specifically in spermatogonia, spermatocytes, and round and elongated spermatids, and the cellular localization of the protein varies from the nuclear compartment in more immature cells to the endosomes in round spermatids. Thus, given its ability to bind androgens and this wealth of structural data, the role of SHBG/ABP in spermatogenesis continues to be its assumed role in either the creation or the maintenance of a special androgen environment required for germ cell differentiation and maturation in the testis and the epididymis, respectively. The problem with this role for SHBG/ABP, however, lies mainly in the fact that androgen receptor presence in germ cells is controversial (Suárez-Quian et al, 1999), suggesting perhaps that if androgens were being delivered to germ cells via SHBG/ABP, then their action would be unlikely to be mediated via a classical androgen receptor mechanism.

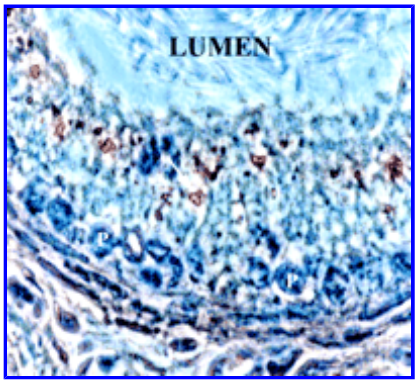

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Figure 3. Sex hormone-binding globulin/androgen-binding protein (SHBG/ABP) immunolocalization in epididymal principal cells. Positive ABP immunostaining in organelles resembling the endosomal compartment of principal cells near their lumen apex is consistent with SHBG/ABP being internalized by receptor-mediated endocytosis by these cells.

The transgenic mice overexpressing rat SHBG/ABP were developed to try to shed additional insight into SHBG/ABP action in spermatogenesis. The transgenic animals exhibit increased expression of rat SHBG/ABP mRNA in Sertoli cells and enhanced DHT-binding activity in plasma and testicular homogenates (Larriba et al, 1995; Esteban et al, 1997a). Interestingly, the first phenotypic abnormality observed was a decrease in fertility. One of the 3 transgenic mouse lines originally developed, for example, was discontinued because of a complete lack of fertility, and the 2 other lines presented a significant reduction in litter size. Continuous matings among pairs of SHBG/ABP heterozygous males and normal females revealed that the problem was a premature onset of reduced fertility, since the number of descendants was almost normal up to 9 months of age, at which time the heterozygous males became sterile (Selva et al, 2000). Regardless of its mechanisms of action, these results confirmed anew that aberrant levels of SHBG/ABP were associated with impaired fertility.

Morphological examination of the transgenic mice testis revealed that all stages of the cycle of the seminiferous epithelium were present but that germ cell differentiation was blocked at the first meiotic division in some tubules (Selva et al, 2000). Further, homozygous mice presented an increased accumulation of pachytene and metaphase spermatocytes with abnormal morphology and localization, and these cells underwent a significant level of apoptosis (Figure 4) (Larriba et al, 1995; Esteban et al, 1997a; Joseph et al, 1997a; Selva et al, 2000). By immunohistochemistry, accumulation of rat SHBG/ABP protein was detected in pachytene spermatocytes and metaphase cells in those tubules presenting arrest of spermatogenesis (Esteban et al, 1997b). Given that the level of SHBG/ABP is low during meiosis in rats (Ritzén et al, 1982), we speculated that an excess of SHBG/ABP in these stages of spermatogenesis led to the meiotic arrest and cellular degeneration detected in the transgenic mice and suggested that excess SHBG/ABP could have a negative control on spermatogenic progression at the level of the first meiotic division of primary spermatocytes (Selva et al, 2000). Given the initial assumption that SHBG/ABP acts by sequestering androgens and that testosterone withdrawal produces apoptosis in pachytene spermatocytes, the results were interpreted to suggest that a decrease in free testosterone caused by the excess SHBG/ABP increase in the transgenic mice could indeed explain the testicular abnormalities. Unfortunately, the levels of total plasma and testicular testosterone in the transgenic mice were not significantly different from those in the normal littermates, although it was not possible to measure the ratio between free and bound testosterone, which is potentially the vital dimension responsible for the deleterious effects. Nevertheless, even if the free testosterone levels were diminished in the transgenic mice, this did not account for the absence of androgen receptor in the germ cells. Thus, the question

remained, how could androgens function directly at the level of germ cells if delivered there by SHBG/ABP?

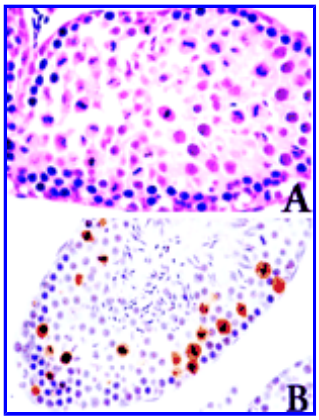

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Figure 4. Hematoxylin-eosin staining and in situ DNA fragmentation analysis in the testis of the rat sex hormone-binding globulin/androgen-binding protein (SHBG/ABP) transgenic mice. Accumulation of meiotic cells with abnormal morphology and localization was frequently observed (**A**). The TUNEL assay showed the presence of an increased number of apoptotic cells compared to nontransgenic littermates. The majority of labeled cells were identified as pachytene and metaphase spermatocytes (**B**).

There is now ample evidence that germ cells express cytochrome P450 aromatase and estrogen receptor ß (Levallet et al, 1998; Saunders et al, 1998). If SHBG/ABP is indeed delivering excess testosterone to germ cells in the transgenic mice, and this testosterone is being converted to estrogen, then one possible effect in these mice is the elevation of intratesticular estrogen levels, a condition known to cause increased germ cell apoptosis (Blanco-Rodríguez and Martínez-García, 1996). It is this proposed "high estrogen" concentration, however, that will lead to the deleterious effects on spermatogenesis, since compelling data suggest that "normal" estrogen levels also have an important role in meiosis. Pachytene arrest of the hypogonadal mice congenitally lacking gonadotropin, for example, achieves qualitatively normal spermatogenesis after estradiol treatment (Ebling et al, 2000). Further, Pentikäinen et al (2000) showed the ability of estradiol to inhibit male germ cell apoptosis induced in vitro by incubating segments of human seminiferous tubules without survival factors. But an equally plausible explanation was that, if SHBG/ABP can indeed bind to estradiol in the rat SHBG/ABP transgenic mice testis, then the excess of SHBG/ABP could result in a net reduction of free estradiol and blockade of meiosis in a stage-specific fashion. To test these 2 possible modes of action, the levels of androgen and estrogen receptors and P450 aromatase were measured in the testis of the rat SHBG/ABP transgenic mice. As expected, while the androgen receptor did not show significant changes, the mRNA levels of P450 aromatase and estrogen receptor ß were increased in the testes of transgenic mice. More significantly, the estrogen receptor ß protein was elevated in the cytoplasm of dying pachytene spermatocytes, suggesting a decrease in the amount of free estradiol and the inability of the estrogen receptor ß to translocate to the nuclei. While these findings are consistent with the proposed mechanism of decreased estrogens acting as a causative agent in negatively affecting spermatogenesis in the transgenic mouse, yet a third possibility is that an SHBG/ABP complex may also exert a direct effect on spermatocyte apoptosis (Selva et al, in preparation). Finally, it is possibly that all 3 scenarios take place simultaneously, but the distinct phenomena are compartmentalized as a function of the cycle of the seminiferous epithelium.

The transgenic mouse models that overexpress the human SHBG/ABP, despite their increased testicular SHBG/ABP mRNA expression, exhibit normal spermatogenesis and fertility (Jänne et al, 1998). As described above, the expression of the human SHBG/ABP in the testis was much higher in transgenic

lines that contain the P1 and PA promoters, and the alternative transcripts replaced the mRNAs corresponding to the secreted form, suggesting that a similar pattern of expression might occur in the human testis (Jänne et al, 1998). In this case, it can be speculated that the testicular-secreted form of SHBG/ABP is dispensable during adulthood in humans because the hepatic-secreted form is able to replace its function.

The presence of SHBG/ABP in semen has been documented in humans (Barahona et al, 1980; Plymate et al, 1981; Oda, 1982; Iosefi and Lewin, 1984; Morvay and Traub, 1984) as well as in other mammalian species (French and Ritzén, 1973; Guerrero et al, 1975; Voglmayr et al, 1977; Jegou and Le Gac-Jegou, 1978; Jegou et al, 1978, 1979; Danzo et al, 1982; Hess et al, 1984; Cheng et al, 1986). In semen obtained from fertile and infertile men and also from vasectomized patients, the existence of ABP was demonstrated by using dextran-coated charcoal and ammonium sulfate precipitation methods and steroid binding. Two groups demonstrated the presence of an SHBG/ABP protein in the seminal fluid of vasectomized patients (Oda, 1982; Iosefi and Lewin, 1984), different from the plasmatic and epididymal form (Oda, 1982). The authors suggested that the protein originates from the prostate and seminal vesicles. Morvay and Traub (1984) identified an SHBG/ABP form in disintegrated sperm cells. Barahona et al (1980) described the presence of SHBG/ABP in blood serum and seminal plasma and also the existence of a smaller protein with androgen-binding capacity in the seminal fluid but not in blood in human samples. Two years earlier, Jegou et al (1978) demonstrated the presence of a specific androgen-binding protein in the seminal plasma of the ram, similar but not identical to epididymal and plasma SHBG/ABP. These authors demonstrated the binding of the seminal protein to DHT, testosterone, and estradiol. In spite of all these data, the direct molecular demonstration of the presence of SHBG/ABP by Western blot in the human semen has not been achieved.

In view of the described observations, many questions arise regarding the expression and the role of SHBG/ABP in human reproduction. We still do not know 1) if the human testis expresses both the secreted and the alternative form of SHBG/ABP, 2) the proportional amount of each, 3) the cellular type responsible for the expression of each, 4) the steroid affinity of each, 5) the binding capacity of the alternative form of SHBG/ABP, 6) their mechanism of action (the existence and nature of the SHBG/ABP receptor and/or other interacting proteins), and 7) the role of the alternative, cytoplasmic form in the male reproductive tissues. Future experiments are required to address these specific points.

In summary, the original role for SHBG/ABP, to serve as a steroid-binding protein that then transports the bound steroid to target cells, remains a viable mechanism of action (Figure 5). In this review, however, we have addressed downstream phenomena to the steroid-binding capability that may help explain potential actions of androgens and estrogens in spermatogenesis. Regardless of what the vital action of SHBG/ABP is ultimately demonstrated to be, there is no doubt that this enigmatic molecule has served an important role in developing proof-of-principle methods for the study of spermatogenesis during the past 25 years.

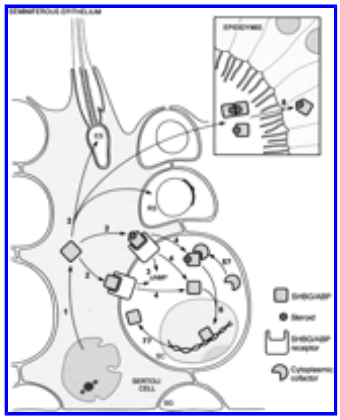

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Figure 5. Model to describe the possible mechanisms of action of sex hormone-binding globulin/androgen-binding protein (SHBG/ABP) in the male reproductive tract. The rat Sertoli cell produces SHBG/ABP (1) (Hagenas et al, 1975; Oke and Suárez-Quian, 1993; Joseph, 1994), which, after binding to steroids or in its free form, interacts with specific plasma membrane receptors on germ cells (2) (Frairia et al, 1992; Porto et al, 1992). (The interaction depicted in the Figure is at a pachytene spermatocyte. However, the SHBG/ABP may also bind to round and elongated spermatids, although there is less evidence for this.) In turn, the receptor could activate a second messenger cascade (3), as was demonstrated in other tissues (Fortunati et al, 1996; Nackla et al, 1997), or, alternatively, the SHBG/ABP protein could be internalized (4) (Gerard et al, 1991, 1994). Once in the germ cell cytoplasm, SHBG/ABP could interact with a cytoplasmic cofactor (5?), although this mechanism of action is not yet demonstrated, and ultimately, the bound complex is delivered to the nucleus, where it interacts with nuclear proteins or DNA (6). It is also possible that the germ cell synthesizes its own SHBG/ABP (7) and in turn functions in an autocrine manner to modulate the intracellular effects of sex steroids or to be delivered into the nuclei (Joseph et al, 1996). The SHBG/ABP protein also migrates to the epididymis, where it is internalized by the principal cells (8) (Feldman et al, 1981; Pelliniemi et al, 1981; Gerard et al, 1998).

Acknowledgments

The authors would like to thank Dr Geoffrey Hammond for his critical reading of the manuscript.

Footnotes

Funding provided by Ministerio de Educación y Ciencia (grant PM98/0161, PM99/0134 y acción integrada con Francia HF1999-0067), Serono-Fundación Salud 2000, Comissionat de Recerca-CIRIT (1999/SGR00231), and by NIH grant HD 23484 to C.A.S.-Q.

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