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Studies on Steroid Binding Proteins in Normal Tissues and Tumor Cell Lines

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Steroid binding proteins bind steroid hormones with high affinity and their function is to carry those hormones in the extracellular compartment. Since their discovery more than fifty years ago, many reports concerning their physicochemical structures and functions have contributed to the better understanding of those proteins. Recent advances in recombinant DNA technology have led to the availability of molecular probes for these proteins, and new approaches have been used to analyse their gene structures as well as the regulation of their synthesis. In the present report, we will review the new findings of the last five years which include the cloning and sequencing of the cDNAs and genes for corticosteroid binding globulin, testosterone estradiol binding globulin and androgen binding protein, as well as the tissue distribution and regulation of their mRNAs in normal tissues and cancer cell lines.

Key words: Steroid hormones, Corticosteroid binding globulin, Testosterone-estradiol binding globulin, Androgen binding protein.

Steroid binding proteins were discovered more than fifty years ago by BRUNELLI who reported that estrogens were bound in part to the globulin fraction of plasma (5). During these five decades, many reports concerning their physicochemical and binding properties as well as their function have contributed to the better understanding of those proteins which carry steroid hormones in the extracellular compartment (3, 24, 31).

Steroid binding proteins bind steroid hormones with high affinity, reversible binding characteristics involving Van der Vaal and hydrophilic forces (33). Other proteins such as albumin also bind steroids but with low affinity and high capacity (36). In the present review we will only refer to three of those high-affinity binding proteins which are Corticosteroid Binding Globulin (CBG), Testosterone-

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Estradiol Binding Globulin (TeBG) and Androgen Binding Protein (ABP).

CBG, also known as transcortin, is synthesized in the liver and transports 90 % of the circulating cortisol. Its production is increased by estrogens and vitamin B12 and decreased by glucocorticoids (4). TeBG is also known as sex-hormone binding globulin and is also synthesized by the hepatocytes. Its role is the transport of testosterone and estradiol in the blood. Estrogens and thyroxin increase and androgens decrease the levels of this protein in plasma (4, 26). ABP is produced by the testicular Sertoli cell and secreted by into the lumen of the seminiferous tubule. Testosterone, following secretion by Leydig cells, traverses the seminiferous epithelium and binds to ABP in the lumen. This protein is then transported to the epididymis where it is internalized by the proximal portion of the caput (1). The function is believed to be the carrier of androgens to the epididymis.

Recent advances in recombinant DNA technology have led to the availability of molecular probes for these proteins, and new approaches have been used to uncover their molecular structures, analyse their gene sequences and study the synthesis and regulation of their mRNAs.

Cloning and analysis of CBG, ABP and TeBG cDNAs and their genes

The cDNA for human CBG (hCBG) was isolated from a human liver cDNA library and sequenced as described (12). When the deduced amino acid sequence of hCBG was compared to the sequences in the protein sequence database of the Protein Identification Resource, there was similarity to several members of superfamily 260 (12). The structural relationship of hCBG to these proteins is summarized in table I. The amino acid overlaps in all the proteins extend over almost their entire sequence. The amino acid identity ranges from 21 to 42 %. This increases to 67-83 % when amino acids with conservative substitutions are included. In spite of the remarkable amino acid sequence similarity between proteins of this group, their obvious functions have diverged to include hormone binding proteins (CBG, TBG), serine protease inhibitors (SERPINs), egg white proteins, and prohormones (12).

When the nucleotide and deduced amino acid sequences of hCBG were compared with published sequences of several other steroid binding proteins, including the glucocorticoid and estrogen receptors, albumin, vitamin D binding protein, α-fetoprotein and TeBG, no significant sequence similarities were observed (12).

One of those selected hCBG cDNA clones was used as probe to screen a human genomic library and identify the hCBG gene (34). The results of this study showed that CBG is encoded by a single gene which spans five exons distributed over approximately 19 Kilobases. In addition, six highly conserved sequence elements, responsible for efficient, liver-specific expression of the mouse albumin gene, are located within the first 200 bp of the 5'-flanking region (34).

Rat ABP (rABP) cDNA clones were also isolated from a rat testis cDNA expression library and sequenced (27,28). With the exception of a single nucleotide difference in the codon of amino acid 317 (G in place of A) which results in a conservative amino acid replacement (Arg in place of His), the results are identical to those reported by Joseph *et al.* (17). Accordingly, the mature monomer would contain 373 amino acids with a predicted molecular mass of 41,183 Da. Previous studies indicate that rABP is composed of two types of monomers designated heavy (H) and light (L), of 45 and 42 kDa, respectively (23). The molecular mass of the cDNA deduced protein is close to that of the L monomer. If the rABP cDNA isolated by this laboratory and that of Joseph *et al.* (17) corresponds to the mRNA

Table I. *Amino acid sequence homology between (corticosteroid binding globulin (CBG), testosterone-estradiol binding globulin (TeBG), serine protease inhibitors (SERPINS), and other members of protein superfamily 260.*

Protein	Residues	Overlap	Identical (%)	Conserved (%)
Human CBG precursor	405	405	100	100
Human TeBG precursor	415	371	42	83
Human α1-antitrypsin precursor	418	366	43	87
Human α1-antichymotrypsin precursor	433	373	44	83
Human antithrombin-III precursor	464	386	31	77
Human plasminogen activator-inhibitor	402	369	27	76
Chicken ovalbumin	385	328	26	74
Chicken gene y protein	388	309	26	76
Chicken gene X protein (fragment)	232	232	28	77
Human angiotensinogen precursor	485	382	21	67

for the L monomer, it is possible that H and L protomers may be encoded by the same mRNA (17, 28). If this is true, then the H monomer is probably a glycosylated form of the L monomer.

The human TeBG (hTeBG) cDNA was also isolated from a human liver cDNA library using the rABP cDNA as probe (13). The amino acid sequence deduced from the cDNA confirms the presence of a leucine at the N-terminus and agrees with the sequence obtained by direct chemical methods (35). When the cDNAs for rABP and hTeBG were compared, they shared a 68 % sequence identity at the amino acid level. It is also known that rABP and hTeBG exhibit a sequence homology with residues 250-634 of protein S, a blood clotting factor (2). Even more surprising is the fact that there appears to be no sequence homology between either rABP or hTeBG and other steroid-binding proteins, including the glucocorticoid (14), estrogen (11) and androgen (6) receptors, albumin (9), vitamin D binding protein (7), and alfa-feto protein (22).

JOSEPH *et al.* (16) used their rABP cDNA to isolate a genomic clone for rABP. This 5.3 Kb fragment contains the entire coding region which is divided into 8 exons. Southern blot analysis of rat genomic DNA indicated that there is a single gene for ABP in the rat. The existence of one gene supports the idea that TeBG produced by fetal rat liver is coded by the same gene (16).

CBG EXPRESSION IN NORMAL TISSUES AND TUMOR CELL LINES

The hCBG cDNA was used as a probe to detect CBG mRNA on RNA blots of poly(A)+RNA prepared from Rhesus monkey tissues. The positive signal of 1.63 Kb corresponding to the CBG mRNA was easily detectable in the liver after a 2 hour exposure and clearly visible in the testis and kidney after 18 hour exposure (12). This mRNA was also found in lung and pituitary but not in heart, skeletal muscle and seminal vesicle of Rhesus monkies (REVENTOS *et al.*, manuscript in preparation).

Total RNA was also extracted from adult rat testis as well as from human testis from a prostate cancer patient who was castrated. Approximately 10 μg of those RNAs were blotted onto nitrocellulose and hybridized with the hCBG cDNA probe (28). As it is presented in fig. 1, after washing the filters at low stringency (2) the hCBG mRNA was identified in the testes of both species. These results con-

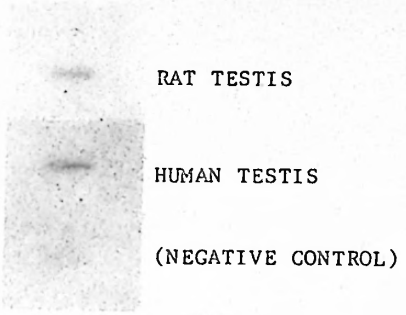

Fig. 1. *RNA blots of rat and human testis.* Total RNA was extracted from testis as described (28), and 10 µg of this were blotted onto nitrocellulose and hybridized with a hCBG cDNA probe. After hybridization the filters were washed at low stringency and exposed to autoradiography (28).

firm our previous finding that CBG is also synthesized in small amount in the testes of Rhesus monkey (12).

The identification of CBG mRNA in tissues other than the liver is an important finding, because it indicates that CBG in glucocorticoid responsive cells (25, 31) may result from low levels of CBG synthesis in addition to or rather than sequestration of the protein from the blood circulation.

The human hepatoma-derived cell line Hep-G2 synthesizes and secretes many different plasma proteins including CBG (18, 19). We have used the hCBG cDNA as probe to identify the CBG mRNA in Hep-G2 cells as well as in SK-HEP-1 cells, another human hepatoma-derived cell line. The cell lines Hep-G2 and SK-HEP-1 as well as their culture conditions have been previously described (10, 19). For these experiments, the cells were grown at 37°C in the presence of 5 % CO_2. When they became confluent, they were washed twice with medium and scraped with a policeman rubber for RNA

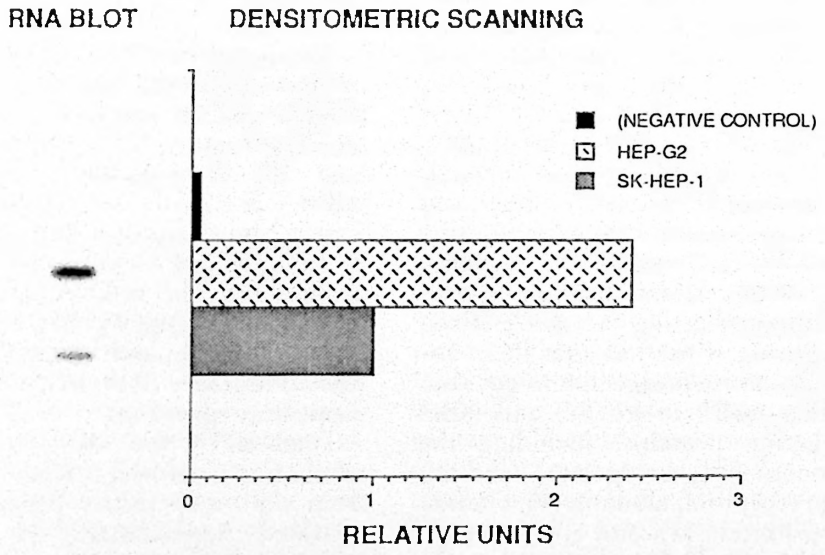

Fig. 2. *RNA blots of human hepatoma cells.* Human hepatoma cells were cultured as described (10, 19), in presence of 5 % CO_2. When cells reached confluency, total RNA was extracted and blotted onto nitrocellulose as described (27). After hybridization the filters were washed at high stringency and exposed to autoradiography (left panel). In order to evaluate the relative amounts of specific mRNA, the X-ray films were analyzed by densitometry (right panel).

extraction. The RNA concentration in the samples was measured by spectrophotometry (OD 260), and same amounts of these RNAs were then transferred to nitrocellulose and hybridized with a labeled CBG cDNA probe. The filter was then washed at high stringency and exposed to autoradiography (fig. 2). The relative amounts of CBG mRNA in both cell lines were quantified by densitometric scanning of the X-ray film (fig. 2). In order to assess the presence on the filter of similar amounts of RNA from both cell lines as well as the negative control, the filters were stripped of the CBG probe and hybridized again (data not shown) with a human actin cDNA probe (28).

The results presented in fig. 2 agree with a previous report from KAHN *et al.* (28) and established the presence CBG mRNA in Hep-G2 cells. We also identified the CBG mRNA in SK-HEP-1 cells (fig. 2) and detected the protein secreted in the culture medium (data not shown). However, Hep-G2 cells contain 2.4 fold more CBG mRNA than SK-HEP-1 cells (fig. 1) and CBG is secreted in the culture medium at the same ratio (data not shown). These results also show that CBG is indeed a product of hepatocytes.

TISSUE DISTRIBUTION AND HORMONAL REGULATION OF rABP mRNA

Our previous studies have established that the rate of rABP secretion is very low in cultures of Sertoli cells isolated from young animals (7-10 days old), but increases in Sertoli cells from 20-day-old animals (29). We extracted RNA from testis, liver and kidney of 10, 20 and 46 day old rats and used the rABP cDNA probe to identify the presence of the rABP mRNA. In agreement with the above mentioned experiments (29), we could not detect rABP mRNA in extracts of testes of 10-day-old rats, but were able to show the presence of this mRNA in the testes of 20 and 46-day-old rats (28). At all three ages,

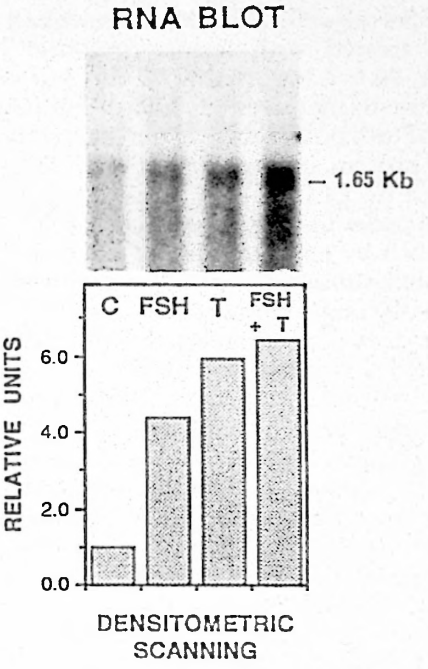

Fig. 3. *RNA blot from testes of control and hormonally treated hypophysectomized rats.* Extracts were fractioned in 1 % agarose formaldehyde electrophoresis, transferred onto nitrocellulose, hybridized with a rABP cDNA probe and exposed to autoradiography (upper panel). In order to estimate the relative amounts of specific rABP mRNA under the different treatments, the X-ray films were quantified by densitometry (lower panel).

rABP mRNA was undetectable in the extracts of liver and kidney, and confirms the tissue specificity of rABP synthesis (27, 28).

It has also been shown by many authors and in different species that testosterone and FSH stimulate the production of ABP by the Sertoli cell in culture and that those hormones also restore *in vivo* the decreased production of rABP in adult rats after hypophysectomy.

We confirmed those hypothesis using the rABP cDNA probe to evaluate the induction of rABP mRNA in 20-day-old

rats Sertoli cells cultured as described (21) and treated with testosterone and FSH (29). As we reported (27), this hormonal treatment increases 2.5 fold the amount of rABP mRNA as well as the production of the protein in the culture medium measured by ELISA.

In order to verify the induction of rABP mRNA by testosterone and FSH after hypophysectomy, the pituitary glands of 26-day-old rats were surgically removed, and four days after surgery, the animals were treated for 7 days with FSH (10 μg/day), testosterone (6 mg/day), both hormones together, or vehicle alone. On the eigth day the animals were killed, and the testes of the animals which received the same treatment were pooled for RNA studies as mentioned above (fig. 3). The levels of rABP mRNA were increased 4.4 fold by FSH, 5.9 fold by testosterone, and 6.4 fold by the combined treatment of FSH and testosterone compared to control untreated hypophysectomized rats (fig. 3).

Discussion

For many years, steroid binding proteins have been thought to have the unique function of being the vehicle of steroid hormones into the vascular compartment. It has also been accepted that these proteins remain in the circulation and are the regulators of the bioavailability of steroids to the cells. It is also believed that only a relatively small fraction of steroids not bound to those proteins will be able to difuse through the plasma membrane and bind to intracellular specific receptors.

Nevertheless, recent data are not consistent with the plasma steroid binding proteins as unique reservoir for hormonal steroids.

Our own and other laboratories (8, 15, 20, 32) have detected specific uptake of iodinated TeBG by epididymal (8) and prostate cells (15), as well as CBG by hepatocytes (20, 32). The specificity of this uptake was examined using competition by excess of unlabelled protein (8, 15, 20, 32). In order to distinguish cell-surface bound from internalized ligand, De Besi *et al.* (8) treated the epididymal cells with trypsin which removes cell-surface bound protein from membrane receptors. These authors found that 70 % of radioactivity was trypsin resistant indicating that an important fraction of cell-associated TeBG was taken inside the cells (8). These studies suggest that TeBG, ABP and CBG enter specific cells by receptor mediated endocytosis.

Once it was evident that there were specific membrane receptors for steroid binding proteins, Rosner *et al.* (30) investigated the ability of hCBG to stimulate adenylate cyclase activity. CBG was found to produce an increase of adenylate cyclase activity assessed by measuring the accumulation of cAMP into the cells (30).

In summary, recombinant DNA technology has provided new understanding of the structure and physiology of steroid binding protein. During the last five years most of cDNAs and genes for those proteins have been cloned and sequenced and their molecular structures accurately determined. These studies have allowed the discovery of striking and unexpected homologies among totally unrelated proteins. Using these cDNAs as probes, many different groups have studied the hormonal regulation of the synthesis of these proteins as well as the tissue distribution of their mRNAs in normal animals and cancer cell lines. Further studies on the development of endocrine dependent tumors might reveal the importance of these proteins as tumor markers and the potential use of DNA probes as diagnostic tools.

Taken together, these new findings suggest that steroid binding proteins are not simply a passive reservoir of hormonal steroids but play a very complex function in the homeostasis of normal and cancer cells.

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Resumen

Las hormonas esteroideas se transportan en el compartimiento extracelular unidas con alta afinidad a proteínas específicas (*Steroid Binding Proteins*). Desde su descubrimiento hace más de 50 años, numerosos trabajos han contribuido a un mejor conocimiento de las estructuras y funciones de esas proteínas. Avances recientes en la tecnología del ADN recombinante han posibilitado nuevas investigaciones moleculares para estas proteínas, con utilización de nuevos enfoques en el análisis de las estructuras génicas y de la regulación de su síntesis. En este trabajo, se revisan los hallazgos de los últimos cinco años, que incluyen la clonación y secuenciación de los cDNAs y de los genes de las globulinas de unión a corticosteroides (*Corticosteroid Binding Globulin*), a testosterona y estradiol (*Testosterone Estradiol Binding Globulin*) y de las proteínas que transportan andrógenos (*Androgen Binding Proteins*), así como su distribución tisular y la regulación de sus mRNAs en tejidos normales y en líneas celulares cancerosas.

Palabras clave: Hormonas esteroideas, Globulinas de unión a corticosteroides, Globulinas de unión a testosterona y estradiol, Proteínas transportadoras de andrógenos.

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