Pergamon

0960-0760(94)00103-0

J. Steroid Biochem. Molec. Biol. Vol. 51, No. 1/2, pp. 115–124, 1994

0960-0760/94 $7.00 + 0.00

Interaction of Sex Hormone-binding Globulin with Plasma Membranes from the Rat Epididymis and other Tissues

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The binding of human sex hormone-binding globulin (hSHBG) to plasma membranes prepared from the adult rat epididymis and other potential target and non-target tissues was examined. Specific binding sites were detected in the epididymis, testis, prostate, skeletal muscle and liver. The first three organs exhibited a higher (K_D approx. 0.1 nM; B_{max} approx. 0.05–0.10 pmol/mg membrane protein, Site I) and a lower (K_D approx. 5 nM; B_{max} approx. 1.0–2.5 pmol/mg membrane protein, Site II) affinity binding site. Only Site I was detected in muscle membranes and only Site II was detected in membranes isolated from liver. Specific binding was not detectable in either spleen or brain. Regional distribution of hSHBG binding sites occurred in the epididymis. Both Site I and Site II were present in the proximal caput and distal cauda. The distal caput and proximal cauda contained only Site II; no specific binding was detected in the corpus. Binding of hSHBG to epididymal membranes was time- and temperature-dependent. The presence of Ca^{2+} did not affect binding. Non-liganded [^{125}I]-labeled hSHBG can bind to both sites in epididymal membranes. The affinity of hSHBG for Site I increased 2-fold when it was complexed with 5α-dihydrotestosterone, testosterone or estradiol. The hSHBG–androgen complex had little effect on Site II versus steroid-free SHBG. However, the affinity of the hSHBG–estradiol complex for these sites was increased 10-fold. Cortisol, which has a low affinity for hSHBG, did not influence its binding to either the higher or lower affinity membrane sites.

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INTRODUCTION

In human and animal blood plasma, sex steroid hormones are largely bound to a specific glycoprotein—SHBG (sex hormone-binding globulin) [1, 2]. Recent studies reveal that recognition systems exist for this protein in plasma membrane preparations of several steroid target tissues [see, for review, Refs 3–5]. These studies suggest the involvement of SHBG in transmembrane transport of steroids and/or signal transduction mechanisms. Another sex steroid binding protein, ABP (androgen-binding protein), a product of the Sertoli cells of the testis [6, 7], has been described in several species including man [8–11]. ABP and SHBG are products of the same gene [12–14], the primary difference between the proteins is post-translational modification due to glycosylation [15]. Comparison of the amino acid sequences of these proteins from human and animal species revealed a high degree of conservation of primary structure [14, 16–17]. ABP is thought to be involved in regulating various aspects of male reproductive function such as spermatogenesis and/or the acquisition of fertilizing ability by sperm. Immunocytochemical [18, 19], autoradiographic [20, 21] and other evidence [22] indicate that ABP is taken up by specific cells in specific regions of the epididymis.

A recent paper by Felden *et al.* [23] presents data indicating that ABP and SHBG labeled with the photoaffinity ligand Δ^6testosterone bind to specific sites in the plasma membrane-enriched fraction of rat epididymal epithelial cells. In our study, we have investigated the binding of SHBG to rat epididymal membranes and to

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Abbreviations: SHBG, sex hormone-binding globulin; ABP, androgen binding protein.

Received 12 Nov. 1993; accepted 3 Jun. 1994.

membranes of other potential target and non-target tissues. The influence of steroid on the interaction of SHBG with plasma membranes was also examined because of the possibility that the binding of liganded versus unliganded protein may effect different biological responses. Since ABP and SHBG are evolutionarily conserved molecules, we considered it likely that the ABP-receptor is similar to the SHBG-receptor, or that the same receptor binds both proteins. The fact that a decapeptide, amino acids 48–57, which appears to be responsible for receptor binding, is present in both human SHBG and rat ABP [24] supports our contention. Further support is provided by the work of Felden *et al.* [23] who observed that the binding characteristics of both of these proteins to rat epididymal membranes were identical. Therefore, because of the greater availability of human SHBG, we used it as the ligand for studying ABP/SHBG binding components in the rat epididymis and other tissues.

MATERIALS AND METHODS

Materials

Steroids, salts, Bolton–Hunter reagent, charcoal, phenylmethylsulphonyl fluoride (PMSF), leupeptin, pepstatin and EDTA were purchased from Sigma (St Louis, MO, U.S.A.). Iodo-Gen was obtained from Pierce (Rockford, IL), sodium [^{125}I]iodide was purchased from New England Nuclear (Boston, MA). Bio-Gel P-6DG and electrophoresis supplies were purchased from Bio-Rad (Richmond, CA).

SHBG preparation

Human SHBG (hSHBG) was isolated from postpartum serum by affinity chromatography on immobilized cortisol [25]. The hSHBG preparation was further purified by chromatography on Superose 12 using an FPLC system (Pharmacia, Piscataway, NJ). The purified SHBG preparation was tested for its ability to bind steroids [7] and was characterized by electrophoresis under native [26] and denaturing [27] conditions. These analyses indicated that the hSHBG preparation was highly purified (greater than 98%) and exhibited the physicochemical characteristics typical of the protein [15]. hSHBG was modified using the Bolton–Hunter method [28]. Excess of unreacted Bolton–Hunter reagent was removed by filtration through Bio-Gel P-6DG. Modified hSHBG was tested for its ability to bind steroids and was labeled with sodium [^{125}I]iodide and Iodo-Gen [29]. Labeled hSHBG had a specific activity of 15–20 μCi/μg and a radiochemical purity of 98% as determined by electrophoresis under native and denaturing conditions and by precipitation with an hSHBG specific antiserum. To obtain labeled and unlabeled hSHBG devoid of steroid, treatment with a 1% dextran-coated charcoal suspension at 37°C for 30 min was performed. [^{3}H]testosterone was added to unlabeled SHBG before charcoal treatment to determine the efficiency of steroid removal.

Membrane isolation

An enriched fraction of epididymal membranes was obtained from whole epididymides of adult rats using differential centrifugation. Frozen (−70°C) epididymides were pulverized in a blender (Warring) and suspended in Krebs–Ringer buffer containing PMSF (0.5 mM), pepstatin (0.1 mM) and leupeptin (0.1 mM) for 4 h at 4°C with stirring to remove endogenous ABP. The washed tissue fragments were sedimented by centrifugation at 10,000 *g* for 15 min. Afterwards, the epididymal preparation was homogenized in Krebs–Ringer buffer using a teflon–glass homogenizer. Nuclear and mitochondrial fractions of the epididymal cells were removed by successive centrifugations at 1000 *g* for 15 min and at 18,000 *g* for 20 min. The membrane enriched fraction was obtained by centrifugation of the 18,000 *g* supernatant at 100,000 *g* for 1 h. The 100,000 *g* membrane pellet was suspended in Krebs–Ringer buffer and kept in 40% glycerol at −20°C until used. Membranes from epididymal segments and from other tissues were prepared in the same manner except that the tissues were minced with scissors prior to homogenization. Examination of the membrane enriched fraction by electron microscopy revealed closed membrane vesicles to be a major component of the preparations. The concentration of protein in membrane preparations was determined using Lowry's method [30].

Binding assay

Details of the binding studies are given in the text and Figure legends. In brief, [^{125}I]hSHBG, alone or together with unlabeled hSHBG, was incubated with membrane preparations. The incubations were conducted for various times at 4 or 37°C. To study the steroid-dependency of the binding reactions, hSHBG was incubated with testosterone, 5α-dihydrotestosterone, estradiol (each 5×10^{-7} M) or cortisol (5×10^{-5} M) and in the absence of steroid prior to its addition to the membrane preparations. After incubation, the membranes were precipitated by centrifugation at 12,000 *g* at 4°C for 10 min. Radioactivity in the pellets was counted using a Beckman gamma counter. In all experiments, samples were assayed in triplicate, the replicates did not differ by more than 10%.

RESULTS

The effect of incubation time and temperature on SHBG binding to rat epididymal membranes

When [^{125}I]hSHBG was incubated with rat epididymal membranes at two temperatures (4 and 37°C) for

various lengths of time, we noted that time- and temperature-dependent binding equilibrium was achieved. The binding reaction reached equilibrium after about 30 min when the incubation was conducted at 37°C [Fig. 1(A)] and between 1 and 3 h when the incubation was conducted at 4°C [Fig. 1(B)]. Maximum binding obtained at both incubation temperatures was similar. Figure 1, curve 1 indicates binding that occurred when membranes were incubated with [^{125}I]hSHBG alone (total binding); curve 2 indicates binding that occurred in the presence of [^{125}I] hSHBG and a 500-fold excess of unlabeled hSHBG ("non-specific" binding); curve 3 is the difference between curves 1 and 2 (specific-binding).

The effect of Ca^{2+} on binding of hSHBG to epididymal membranes

To study the influence of Ca^{2+} on binding of hSHBG to rat epididymal membranes, incubation of iodinated hSHBG with membrane preparations was performed in the presence and absence of Ca^{2+}. In addition, incubations were performed in the absence of Ca^{2+} and in the presence of 20 mM EDTA to chelate divalent cations. Two concentrations of hSHBG were used in these studies so that both Site I and Site II (see below) could be estimated. Our data (Fig. 2) indicated no Ca^{2+}-dependence for binding to either site.

Steroid dependence of SHBG binding to epididymal membranes

Further studies on the binding of hSHBG to rat epididymal membranes were performed at 4°C. This temperature was selected to avoid potential complications from protease activity, transport of hSHBG into membrane vesicles, or from receptor–ligand internalization that might occur at 37°C. Steroid-free hSHBG and hSHBG complexed with steroids that might be present in the epididymis were used as ligands. In initial experiments, steroid-free labeled/unlabeled SHBG and labeled/unlabeled SHBG complexed with testosterone or estradiol were used. In all cases saturation of a limited number of binding sites occurred [Fig. 3(A–C)]. However, another component that was not saturated by the amount of labeled hSHBG added was also present [Fig. 3(A–C)]. These data indicated that hSHBG was interacting with two classes of binding sites: one of low capacity (Site I) and the other of high capacity (Site II). Scatchard [31] analysis of the low capacity portion of the saturation curves indicated that binding to a high affinity component had occurred [Fig. 3(D) and Table 1]. The binding component in the membranes had a greater affinity for the hSHBG–steroid complexes than for steroid-free hSHBG [Table 1, Fig. 3(D)].

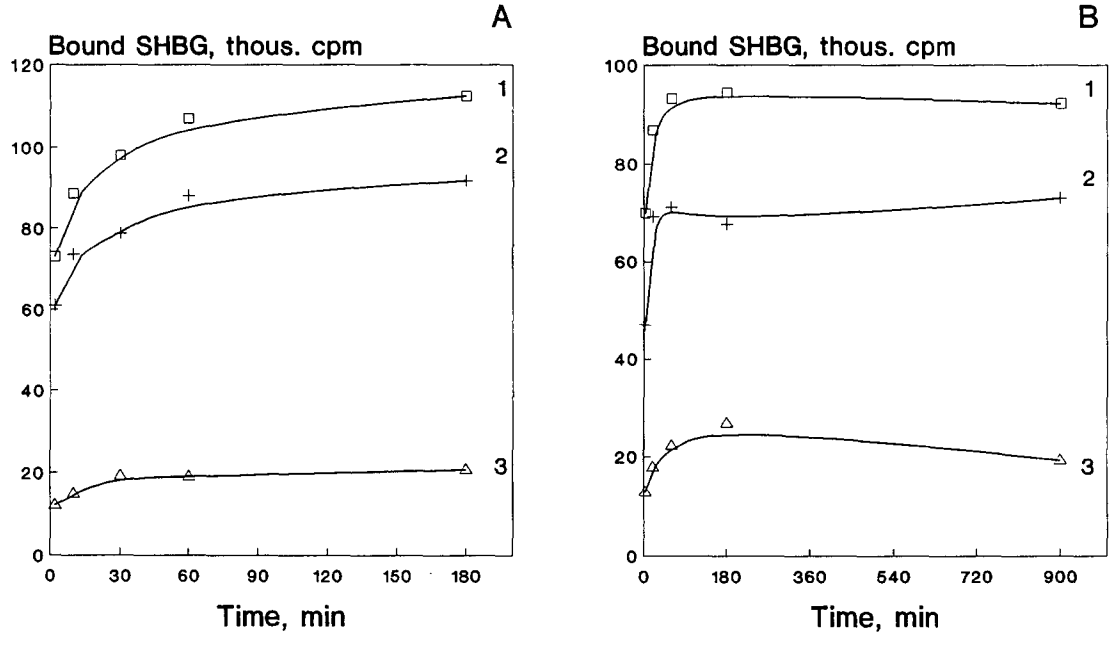

Fig. 1. Time-dependence of SHBG binding to epididymal cell membranes. SHBG, labeled SHBG and epididymal plasma membranes were prepared as described in Materials and Methods. [^{125}I]SHBG (500,000 cpm or 5×10^{-10} M) was incubated with epididymal plasma membranes (about 0.2 mg membrane protein) suspended in Krebs–Ringer buffer for 2, 10, 30, 60 and 180 min at 37°C (A) or for 3, 20, 60, 180 min and 15 h at 4°C (B). In parallel, the control samples were incubated in the presence of [^{125}I]SHBG and 2.5×10^{-7} M unlabeled SHBG. After incubation, membranes were precipitated by centrifugation (12,000 *g*, 10 min). All samples were in triplicate. Curve 1, binding in the absence of unlabeled SHBG (total binding); curve 2, binding in the presence of unlabeled SHBG (non-specific binding); curve 3, specific binding calculated by subtracting curve 2 from total binding. The results of a typical experiment are shown.

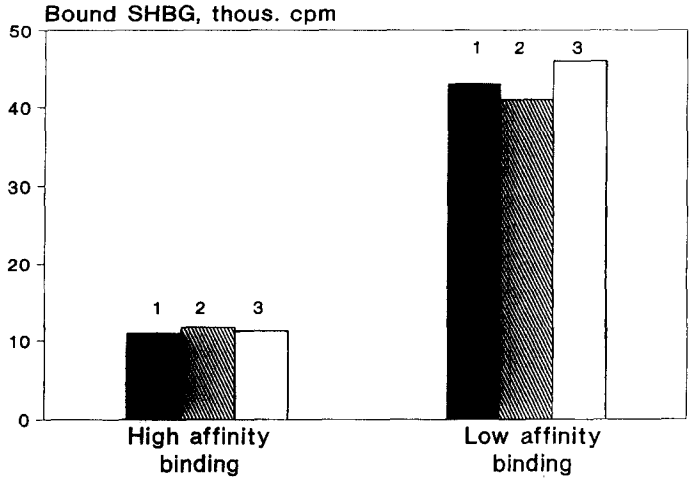

Fig. 2. Influence of Ca^{2+} on binding of SHBG to epididymal cell membranes. Labeled hSHBG (500,000 cpm for the estimation of Site II binding and 50,000 cpm for the estimation of Site I binding) was incubated with epididymal membranes in the absence (total binding) and in the presence (non-specific binding) of 5×10^{-7} M unlabeled SHBG (See Materials and Methods for details). This experiment was performed in the absence of Ca^{2+} (bar 1); in the presence of 2 mM $CaCl_2$ (bar 2); and in the absence of Ca^{2+}, but in the presence of 20 mM EDTA (bar 3). The bars represent only specific binding.

To study the interaction of hSHBG with Site II, assays were performed using a fixed concentration of labeled hSHBG and increasing concentrations of unlabeled hSHBG. Specific, saturable binding of steroid free hSHBG and hSHBG complexed with testosterone or estradiol occurred [Fig. 4(A–C)]. Binding to these sites was also of high affinity [Fig. 4(D), Table 1], but the affinity constant was one to two orders of magnitude less than that of Site I. The affinity of Site II for the estradiol–hSHBG complex was 6-fold higher than that for the testosterone–hSHBG complex and 10-fold higher than that for steroid-free hSHBG [Fig. 4(D), Table 1].

We also studied the binding of hSHBG complexed with 5α-dihydrotestosterone and cortisol to epididymal membranes. The hSHBG–5α-dihydrotestosterone complex exhibited the same affinity as the hSHBG–testosterone complex for both binding sites (Table 1). The hSHBG–cortisol complex interacted with Site I with a 10-fold lower affinity than testosterone, estradiol or 5α-dihydrotestosterone complexes. Its binding to Site II was similar to the binding of steroid-free hSHBG (Table 1). In all experiments, using different epididymal membrane preparations, the concentration of binding sites estimated from Scatchard plots was about 0.05–0.1 pmol/mg membrane protein for Site I and about 1.0–2.5 pmol/mg membrane protein for Site II.

Distribution of hSHBG binding sites in the epididymis

Since the epididymis is divided into structurally and functionally distinct regions [32] and since uptake of ABP is restricted to specific portions of the duct [18–22], we performed studies to determine whether ABP/SHBG binding sites were topographically distributed. Our experiments showed that Site I and Site II for androgen-liganded hSHBG are differentially distributed in the rat epididymis (Fig. 5). The proximal caput and distal cauda contained both sites at similar concentrations (Site I, approx. 0.07 pmol/mg membrane protein; Site II, approx 0.8 pmol/mg membrane protein). Only Site II could be detected in the distal caput and proximal cauda; no specific binding of hSHBG occurred to membranes isolated from the corpus. When binding data were analyzed according to Scatchard [31], Site I in the proximal caput and distal cauda had essentially the same apparent equilibrium dissociation constant (K_D 0.08×10^{-9} M, proximal caput; 0.09×10^{-9} M, distal cauda). Site II in these segments had a K_D of approx. 2×10^{-9} M.

Presence of hSHBG binding sites in other rat tissues

The binding of unliganded hSHBG to plasma membranes isolated from the testis, prostate, skeletal muscle, liver, brain and spleen was examined. As in the epididymis, both Site I and Site II were present in testis and prostate plasma membranes (Fig. 6). The dissociation constants of the interaction of hSHBG with these tissues were similar (Table 2). Only Site I was detected in muscle and only Site II was detected in liver plasma membranes (Fig. 6, Table 2). Muscle had the greatest concentration of Site I and the prostate had the highest concentration of Site II (Fig. 6). No specific binding of hSHBG to rat spleen or brain membranes over the concentration range of 0.01–100 nM was detected.

DISCUSSION

This study was performed using human hSHBG that was labeled on phenyl rings created by modification of

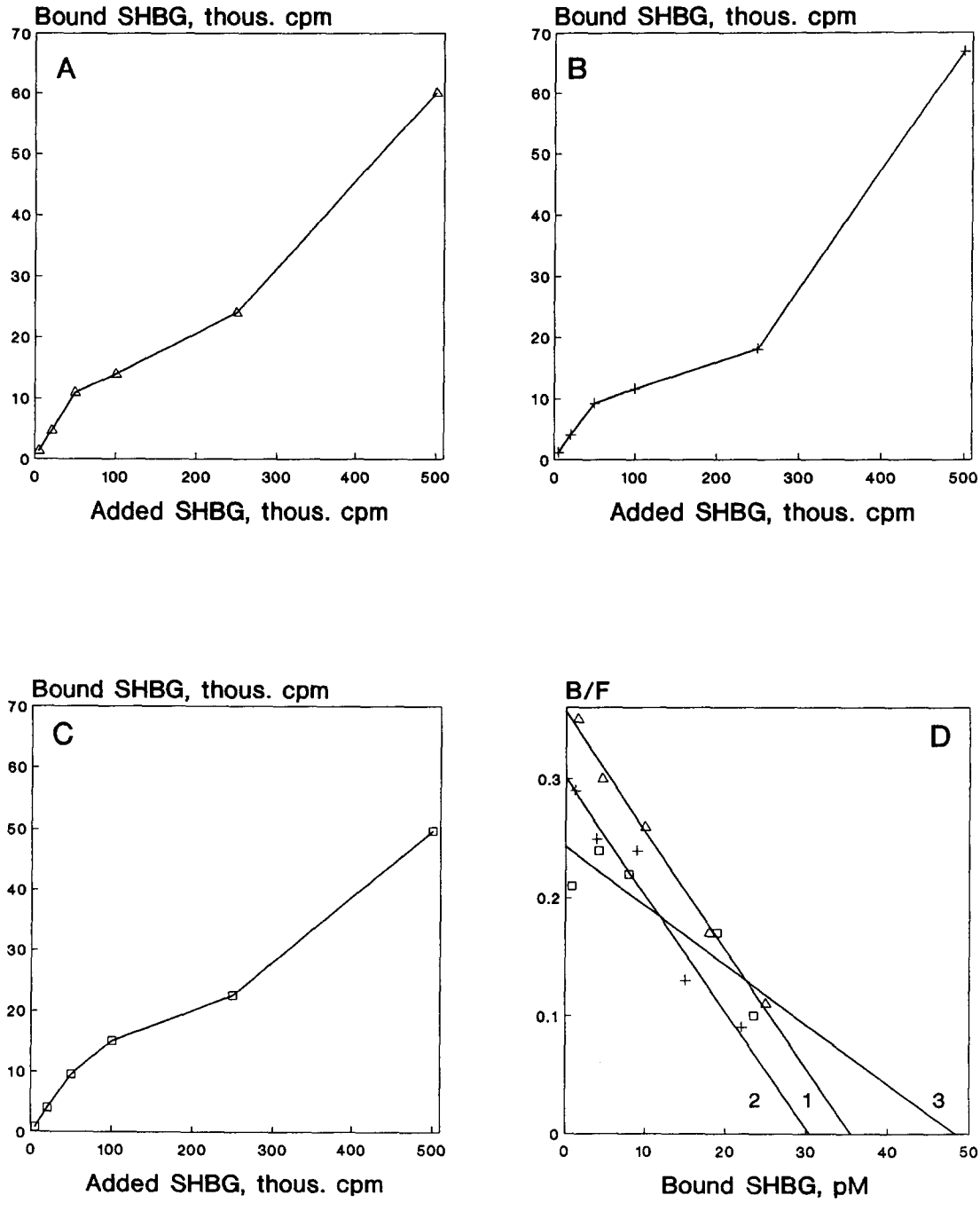

Fig. 3. High affinity (Site I) binding of SHBG to epididymal membranes. Increasing concentrations ($5–500 \times 10^{-12}$ M) of [^{125}I]hSHBG were incubated with membrane preparations in the absence and in the presence of an excess (2.5×10^{-7} M) of unlabeled hSHBG. Labeled and unlabeled hSHBG preparations were stripped of possible endogenous steroid by treatment with charcoal. Equilibration of steroid with hSHBG was done prior to the addition of the membrane suspension to the samples. Saturation curves show low (Site I) and high (Site II) capacity binding of the hSHBG–testosterone complex (A), hSHBG–estradiol complex (B) and steroid-free hSHBG (C) to membranes. (D) Scatchard plots [31] were calculated from the saturation data. The molar concentration of hSHBG added at each point was determined as was the concentration of hSHBG that was bound. The amount of bound hSHBG at each point was subtracted from the total hSHBG added to obtain the concentration of free hSHBG. The bound/free (B/F) ratio was calculated and plotted against the concentration of bound hSHBG to yield to Scatchard plot. A least squares regression line was fitted to the data points. The K_D was obtained by dividing 1 by the slope of the line. Curve 1, hSHBG–testosterone complex; curve 2, hSHBG–estradiol complex; and curve 3, steroid-free hSHBG. The last point of each saturation curve was not used to calculate the Scatchard plots since it does not represent binding to the same site. The data from a typical experiment are shown.

Table 1. Influence of steroid on the affinity of SHBG-membrane interactions

Steroid	Dissociation constant (nM)	
	Site I	Site II
None	0.13 ± 0.02	11.0 ± 1.4
Testosterone	0.07 ± 0.01	6.2 ± 1.2
Estradiol	0.06 ± 0.01	0.97 ± 0.09
5α-Dihydrotestosterone*	0.08	5.0
Cortisol*	0.67	14

*Data from a single experiment are presented for these complexes. Affinity constants were calculated from Scatchard plots [31], obtained as described in Figs 3 and 4 (analogous experiments were performed for SHBG-5α-dihydrotestosterone and SHBG–cortisol complexes, saturation curves and Scatchard plots for these experiments are not shown). Reported values are mean ± SEM from 3 experiments with different membrane preparations.

hSHBG with the Bolton–Hunter reagent [29]. Modified hSHBG has the same affinity for steroids as native hSHBG and reacts with a specific monoclonal antibody [33] (data not shown). Thus, we inferred that modified hSHBG should behave like the native protein in our studies. The association of hSHBG with epididymal membranes occurred rapidly at both 37 and at 4°C. These results are similar to those of Felden *et al.* who showed that the binding of photoaffinity-labeled ABP [34] to rat germ cells [35] and epididymal [23] membranes reaches a maximum in 30–40 min at both 4 and 37°C. In contrast to the rapid association of hSHBG/ABP to epididymal membranes, Rosner *et al.* [36] have shown that association with the solubilized receptor from human prostate occurs slowly and they noted little binding at 4°C. The reasons for the differences observed are not obvious, but tissue differences in the receptor kinetics cannot be excluded.

We did not observe Ca^{2+} dependence for binding of hSHBG to either the high (Site I) or lower (Site II) affinity binding sites of rat epididymal membranes. Our experiments were conducted in both Ca^{2+}-free medium and in medium containing EDTA to chelate potential Ca^{2+} ions. Felden *et al.* [23] reported that binding of ABP to epididymal membranes was Ca^{2+}-dependent, but they did not show a direct influence of Ca^{2+} on binding, but, rather, showed that binding was inhibited in the presence of EDTA.

When the binding of [^{125}I]hSHBG was examined, we noted that 60–70% of the binding was not inhibited by a large excess of unlabeled hSHBG. An explanation for this high degree of non-specific binding is not readily available, however, this phenomenon has also been observed by others [37]. When specific binding was analyzed by the method of Scatchard [31] we determined that two classes of sites for [^{125}I]hSHBG were present in membrane preparations: Site I had a K_D of approx. 0.1 nM and Site II had a K_D of approx. 5 nM. Site I binds both steroid liganded and unliganded hSHBG, but liganded hSHBG consistently binds with a 2-fold higher affinity than unliganded hSHBG. hSHBG liganded with its natural ligands testosterone, dihydrotestosterone or estradiol binds to Site I with the same affinity. Therefore, if this system is responsible for steroid uptake by cells, uptake would be dependent on the androgen/estrogen ratio outside of the cell. If the recognition system is part of a signal transduction mechanism, specificity may be conferred by local steroid concentration as well as post receptor events that are specified by the nature of the target cell. Since the concentration of androgens in the epididymis is high, we presume that androgen-complexed ABP is the biologically active form in that organ. The possibility that unliganded ABP competes for the same binding site as liganded ABP could provide an additional regulatory mechanism.

Site II has a similar affinity for unliganded hSHBG and for hSHBG complexed with cortisol or androgens, however, its affinity for hSHBG complexed with estradiol is 5–10-fold higher. Existence of this site may be related to a special role for estradiol in epididymal cells or, in contrast, may serve as a mechanism protecting cells from high estradiol concentrations and be a clearance mechanism for this steroid. Although referred to as a lower affinity site, Site II has an affinity for complexed and uncomplexed hSHBG that is in the nanomolar range and, therefore, is likely to be physiologically relevant. Two binding sites for ABP/hSHBG have been identified in membrane preparations from the human prostate [36], epididymis [23] and other tissues [38]. The significance of two high affinity binding sites for hSHBG in rat epididymal membranes and in other tissues is not clear, but it is possible that each site exists in a different population of cells and subserves a separate physiological role. For example, Site I may constitute the receptor of a signal transduction system and Site II may be involved in steroid transport and/or metabolism.

Rosner's group has provided evidence that only unliganded hSHBG binds to solubilized human prostatic membranes and that steroid subsequently binds to the hSHBG–receptor complex [39]. In contrast, we show that steroid-liganded hSHBG can bind to receptor in rat epididymal membranes. This observation is supported by data from other laboratories. For example, liganded ABP clearly binds to epididymal [23] and germ cell [35] membranes since the probe used to identify the binding sites was made by covalently linking a steroid to the binding site of ABP. Studies from Strel'chyonok's group [40–41] indicate that the prior interaction of estrogens with hSHBG is required to enable it to bind to endometrial membranes. They also showed that the hSHBG-testosterone complex binds to placental membranes [42]. In other studies [43–44], binding of steroid transport proteins to cell membranes in the apparent absence of steroid was shown. Our principal conclusion from this survey is that both liganded and unliganded hSHBG can bind to sites in non-solubilized membranes from various tissues. Solubilization of the binding sites

may interfere with binding of liganded hSHBG to the sites. This latter supposition is supported by the data of Fortunati *et al.* [38] who showed that estradiol inhibits the binding of hSHBG to solubilized sites in the endometrium while it had no effect on membrane bound sites.

Our demonstration of hSHBG binding sites in the proximal caput of the epididymis correlates well with

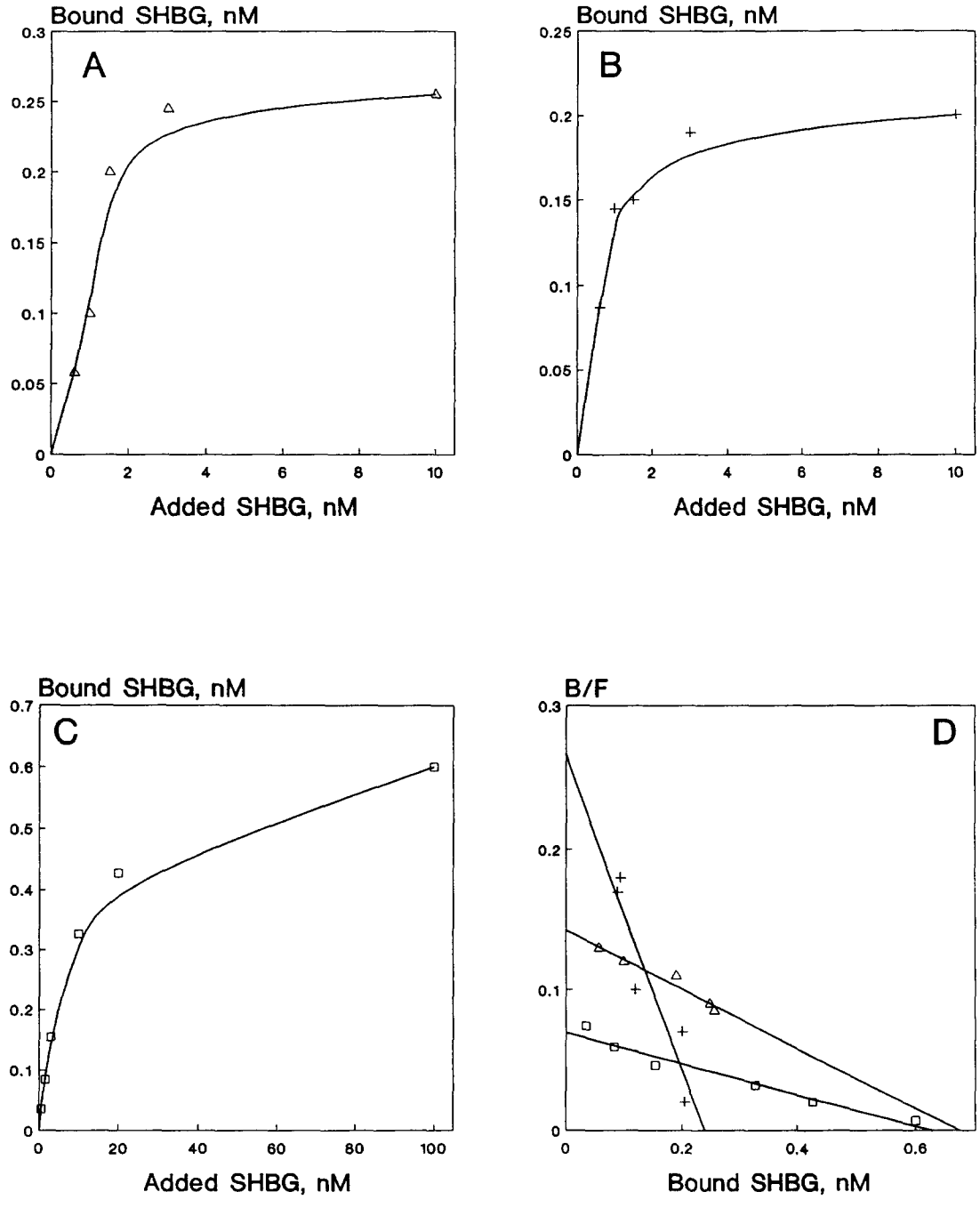

Fig. 4. Lower affinity (Site II) binding of hSHBG to epididymal membranes. [^{125}I]hSHBG (500,000 cpm, 5×10^{-10} M) was incubated with the membranes in the presence of increasing concentrations of unlabeled hSHBG (0–2×10^{-6} M). The saturation data were plotted according to the method of Scatchard [31] and the affinity constants were determined graphically using the procedure of Rosenthal [51]. Steroid-free hSHBG or hSHBG complexed with one of the steroids studied was used in this experiment. Saturation curves for the interaction of the hSHBG–testosterone complex (A), hSHBG–estradiol complex (B) and steroid-free hSHBG (C) with epididymal membranes are shown. For the experimental set with steroid-free hSHBG, a 2-fold higher concentration of membranes was used. (D) Scatchard plots [31] calculated from the saturation data (for details see Fig. 3): hSHBG–testosterone complex (△), hSHBG–estradiol complex (+) and steroid-free hSHBG (□). Results of a typical experiment are plotted.

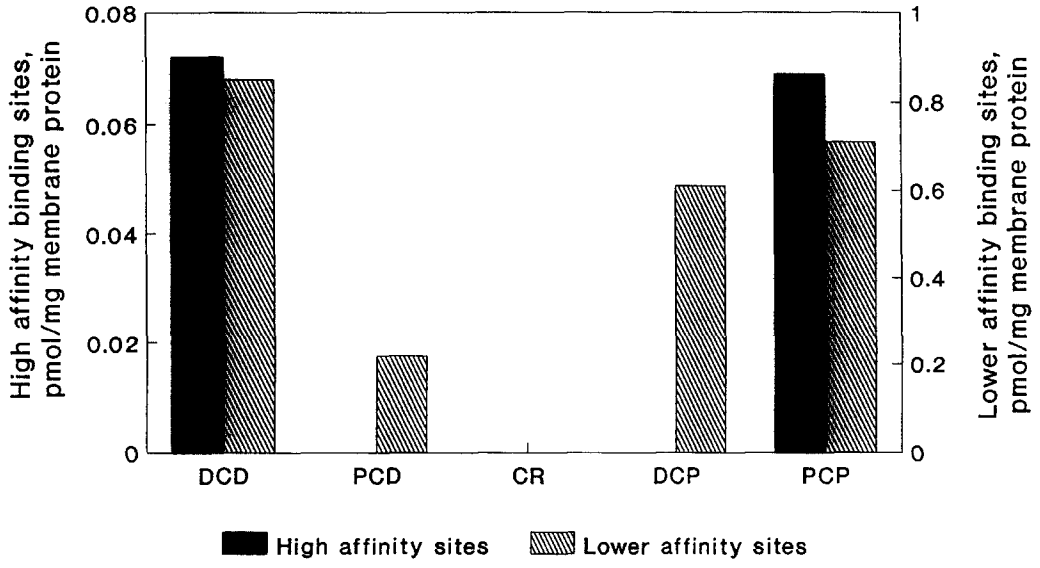

Fig. 5. Distribution of hSHBG binding sites in the epididymis. The epididymides of 10 adult (300–350 g) rats were divided into proximal caput (PCP), distal caput (DCP), corpus (CR), proximal cauda (PCD) and distal cauda (DCD) and plasma membranes were isolated from the pooled segments. The membranes were incubated with 1×10^{-11}–5×10^{-9} M [^{125}I]hSHBG which had been equilibrated with 1×10^{-7} M 5α-dihydrotestosterone in the presence or absence of 5×10^{-7} M unlabeled hSHBG. Binding was determined as indicated in Methods. Dissociation constants and binding site concentrations were determined from Scatchard plots [31].

localization and uptake studies demonstrating the presence of ABP within cells of this region of the epididymis [18–22]. Our finding that the distal cauda had an equivalent concentration of binding sites was unexpected since none of the above studies reported uptake of ABP by this epididymal segment. A physiological relevance for hSHBG binding sites in the cauda, however, cannot be excluded since ABP is present throughout the epididymis in the rat [45] and other species [46]. The highly specific distribution of binding sites along the epididymis supports the contention that these binding sites are involved in mediating specific biological functions along the duct. Our inability to detect hSHBG binding sites in the corpus may be owing to

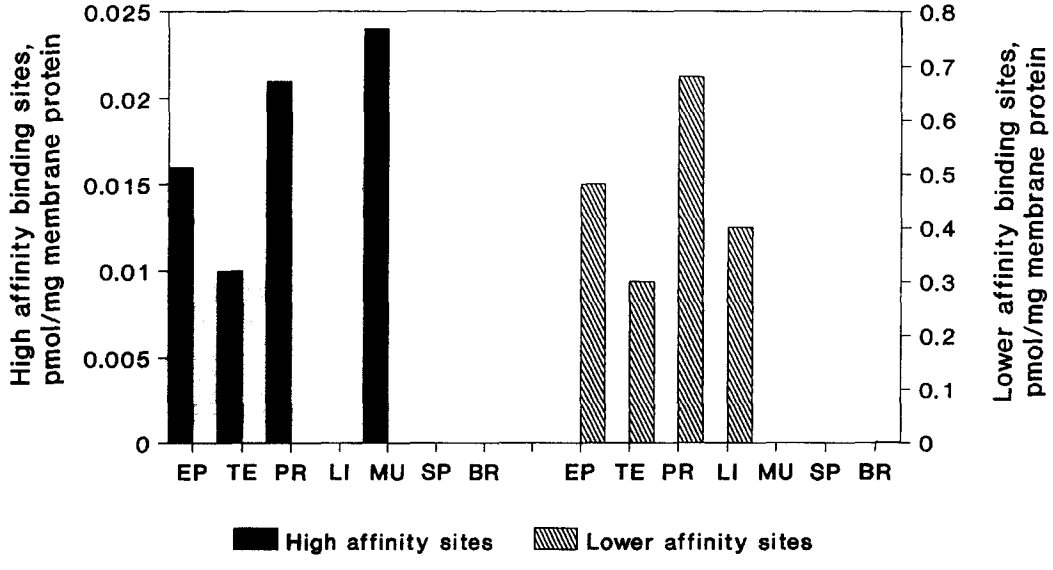

Fig. 6. Distribution of hSHBG binding sites in various rat tissues. Plasma membranes isolated from the epididymis (EP), testis (TE), prostate (PR), liver (LI), muscle (MU), spleen (SP) and brain (BR). Membranes were incubated with 1×10^{-11}–2×10^{-9} M [^{125}I]hSHBG alone and in presence of 5×10^{-7} M unlabeled hSHBG. Binding was determined as indicated in Methods. Dissociation constants and binding site concentrations were determined from Scatchard plots [31].

Table 2. Affinity of membranes isolated from various tissues for SHBG

Tissue	Dissociation constant (nM)	
	Site I	Site II
Epididymis	0.14	4.9
Testis	0.09	4.0
Prostate	0.21	6.7
Muscle	0.30	—
Liver	—	2.9

Affinity constants were calculated as described in Figs 3 and 4.

the absence of such sites (and consequently a lack of a physiological role in ABP in this segment of the duct) or to a much lower concentration of sites than in the other segments.

The rat prostate, like that of the human [38–39] contains high and lower affinity plasma membrane binding sites for hSHBG. However, the affinities of these sites for hSHBG in the rat prostate are about 10 and 30 times, respectively, greater than those reported for human prostate. The concentrations of high and lower affinity binding sites in the rat prostate are about one-fiftieth those in the human prostate. In contrast to the work of Porto *et al.* [44], we find two classes of hSHBG binding sites in rat testis plasma membranes having affinities 500 and 10 times greater, respectively, than that which they report. Felden *et al.* [35] reported the existence of one type of binding site for hSHBG in rat germ cells with a dissociation constant of 1.72 nM. This is similar to Site II we describe in the testis. Absence of Site I in their experiments may be due to the use of isolated germ cells versus the whole organ.

In our study, skeletal muscle exhibited Site I binding sites for hSHBG, but lacked the Site II binding site. These data are in contrast to those of Frairia *et al.* [47] who noted no specific hSHBG binding to muscle. Androgens have positive anabolic and other effects on muscle [48]. Although most of these effects are probably mediated through the androgen receptor in muscle [48], it is possible that the hSHBG binding site may be involved in mediating some androgenic effects in this tissue. The Site II present in liver is not the asialoglycoprotein receptor [49] since binding of desialylated hSHBG to liver membranes has a dissociation constant estimated at 1–10 μM (data not shown). Since the liver is the site of SHBG synthesis, it is not surprising that receptors for the protein would be present in the organ.

The distribution of Sites I and II among the rat tissues does not appear to be related to differential recovery of membrane components from the tissues. Electron microscopic analysis of the preparations used indicated that the predominant component in them was plasma membranes. Membrane preparations from the cauda of the epididymis and from muscle, had the lowest level of non-plasma membrane components. Nevertheless, the cauda showed the presence of both binding sites while the muscle exhibited only the higher affinity site. These data would suggest that the presence of two sites is not related to binding to different membrane components within the preparations. Further studies, however, are required to clarify this issue.

The concentration of androgen-binding proteins in the blood of adult rats is low [50], while during early postnatal development ABP levels in the circulation are high [50]. Whether the ABP/SHBG binding sites in peripheral tissues of the adult rat are functionally active or whether they only function during early development remains to be elucidated. However, in animals that have androgen-binding proteins in the general circulation throughout life, such peripheral tissues could clearly be targets for ABP/SHBG.

Acknowledgements—The authors wish to thank Dr Loren Hoffman (Electron Microscopy Core Laboratory of the Center for Reproductive Biology Research, Vanderbilt University School of Medicine) for characterization of membrane preparations and Pamela Reed for excellent secretarial assistance. These studies were supported by NIH grant HD-13813 and a grant from the Andrew W. Mellon Foundation. Dr N. I. Krupenko is supported by a fellowship from the Rockefeller Foundation. Dr S. A. Krupenko is supported in part by a grant from the Vanderbilt University Research Council. The Center for Reproductive Biology Research is supported by NIH grant HD-05797.

REFERENCES

1. Westphal U.: *Steroid–Protein Interactions*. Part I. Springer-Verlag, New York (1971).
2. Westphal U.: *Steroid–Protein Interactions*. Part II. Springer-Verlag, New York (1986).
3. Rosner W.: The functions of corticosteroid-binding globulin and sex hormone-binding globulin: recent advances. *Endocrine Rev.* 11 (1990) 80–91.
4. Avvakumov G. V. and Strel'chyonok O. A.: Specific steroid-binding glycoproteins of human blood plasma: novel data on their structure and function. *J. Steroid Biochem.* 35 (1990) 519–534.
5. Rosner W., Hryb D. J., Khan M. S., Nakhla A. M. and Romas N. A.: Sex hormone-binding globulin. Binding to cell membranes and generation of a second messenger. *J. Andrology* 13 (1992) 101–106.
6. Steiberger A., Heindel J. J., Lindsey J. N., Elkington J. S. H., Sanborn B. M. and Steinberger E.: Isolation and culture of FSH responsive Sertoli cells. *Endocr. Res. Commun.* 2 (1975) 261–267.
7. Schmidt W. N., Taylor C. A., Jr. and Danzo B. J.: The use of a photoaffinity ligand to compare androgen-binding protein (ABP) present in rat Sertoli cell culture media with ABP present in rat epididymal cytosol. *Endocrinology* 108 (1981) 786–794.
8. Danzo B. J., Orgebin-Crist M-C. and Toft D. O.: Characterization of a cytoplasmic receptor for 5α-dihydrotestosterone in caput epididymidis of intact rabbits. *Endocrinology* 92 (1973) 310–317.
9. Carreau S., Drosdowsky M. A. and Curot M.: Age related effects on androgen-binding protein (ABP) in sheep testis and epididymis. *Intl. J. Androl.* 2 (1979) 49–67.
10. Jegou B. and Gac-Jegou F.: An androgen-binding protein in the seminal plasma of some mammalian species. *J. Endocr.* 77 (1978) 267–268.
11. Hsu A. F. and Troen P.: An androgen-binding protein in testicular cytosol of human testis: comparison with human testosterone estrogen binding globulin. *J. Clin. Invest.* 61 (1978) 1611–1619.
12. Hammond G. L., Underhill D. A., Smith C. L., Coping J. S., Harley M. J., Musto N. A., Cheng C. Y. and Bardin C. W.: The cDNA-deduced primary structure of human sex hormone-bind-

ing globulin and location of its steroid-binding domain. *FEBS Lett.* **215** (1987) 100–104.
13. Que B. G. and Petra P. H.: Characterization of a cDNA coding for sex steroid-binding protein of human plasma. *FEBS Lett.* **219** (1987) 405–409.
14. Joseph D. R., Hall S. H. and French F. S.: Rat androgen-binding protein: evidence for identical subunits and amino acid homology with human sex hormone-binding globulin. *Proc. Natn. Acad Sci. U.S.A.* **84** (1987) 339–343.
15. Danzo B. J., Black J. H. and Bell B. W.: Analysis of the oligosaccharides on androgen-binding proteins: implications concerning their role in structure/function relationships. *J. Steroid Biochem. Molec. Biol.* **40** (1991) 821–831.
16. Walsh V. A., Titani K., Takio K., Kumor S., Hayes R. and Petra P. H.: Amino acid sequence of the sex steroid binding protein of human blood plasma. *Biochemistry* **25** (1986) 7584–7590.
17. Hammond G.: Molecular properties of corticosteroid binding globulin and the sex-steroid binding proteins. *Endocrine Rev.* **11** (1990) 65–79.
18. Feldman M., Lea O. A., Petrusz P., Tress L. L., Kierszenbaum A. L. and French F. S.: Androgen-binding protein purification from rat epididymides, characterization, and immunological localization. *J. Biol. Chem.* **256** (1981) 5170–5175.
19. Pelliniemi L. J., Dym M., Gunsalus G. L., Musto N. A., Bardin C. W. and Fawcet D. W.: Immunochemical localization of androgen-binding protein in the male reproductive tract. *Endocrinology* **108** (1981) 925–931.
20. Gerard A., Khanfri J., Guenant J. L., Fremont S., Nicolas J. P., Grigon G. and Gerard H.: Electron microscope radioautographic evidence of *in vivo* androgen-binding protein internalization in the rat epididymis principal cells. *Endocrinology* **122** (1988) 1297–1307.
21. Gerard A., Egloff M., Gerard M., El Harate A., Domingo M., Guenant J. L., Dang C. D. and Degrelle H.: Internalization of sex steroid-binding globulin in the monkey epididymis. *J. Molec. Endocr.* **5** (1990) 239–251.
22. Veeramachaneni D. N. R. and Amann R. P.: Endocytosis of androgen binding protein, clusterin, and transferrin in the efferent ducts and epididymis of the ram. *J. Androl.* **12** (1991) 288–294.
23. Felden F., LeHaup B., Fremont S., Bouguerne R., Egloff M., Nicolas J. P., Grignon G. and Guenant J. L.: The plasma membrane of epididimal epithelial cells has a specific receptor which binds to androgen-binding protein and sex steroid-binding globulin. *J. Steroid Biochem. Molec. Biol.* **42** (1992) 279–285.
24. Khan M. S., Hryb D. J., Hashim G. A., Romas N. A. and Rosner W.: Delineation and synthesis of the membrane receptor binding domain of sex hormone-binding globulin. *J. Biol. Chem.* **265** (1990) 18,362–18,365.
25. Strel'chyonok O. A., Survilo L. I., Tsapelik G. Z. and Sviridov O. V.: Purification and physico-chemical properties of human plasma sex steroid-binding globulin. *Biokhimiya* **48** (1983) 756–762.
26. Davis B. J.: Disk electrophoresis—II Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121** (1964) Pt. 2, 404–427.
27. Laemmli U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227** (1970) 680–685.
28. Bolton A. E. and Hunter W. M.: The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. *Biochem. J.* **133** (1973) 529–539.
29. Fraker P. J. and Speck J. C.: Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3α,6α-diphenylglucoluril. *Biochem. Biophys. Res. Commun.* **80** (1978) 849–857.
30. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
31. Scatchard G.: The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51** (1949) 660–672.
32. Orgebin-Crist M. C., Danzo B. J. and Davies J.: Endocrine control of the development and maintenance of sperm fertilizing ability in the epididymis. In: *Handbook of Physiology* (Edited by D. W. Hamilton and R. O. Greep). American Physiological Inc. Washington, DC., Vol. 5, Section 7 (1975) pp. 319–338.
33. Kovacs W. J., Bell B. W., Turney M. K. and Danzo B. J.: Monoclonal antibodies to rat androgen-binding protein recognize both of its subunits and cross-react with rabbit and human testoserone-binding globulin. *Endocrinology* **120** (1988) 2639–2647.
34. Taylor C. A. Jr., Smith H. E. and Danzo B. J.: Photoaffinity labeling of rat androgen-binding protein. *Proc. Natn. Acad. Sci. U.S.A.* **77** (1980) 234–238.
35. Felden F., Guenant J. L., Ennya A., Gerard A., Fremont S., Nicolas J. P. and Gerard H.: Photoaffinity labelled rat androgen-binding protein and human sex hormone steroid-binding protein bind specifically to rat germ cells. *J. Molec. Endocr.* **9** (1992) 39–46.
36. Hryb D. J., Khan M. S., Romas N. A. and Rosner W.: Solubilization and partial characterization of sex hormone-binding globulin receptor from human prostate. *J. Biol. Chem.* **264** (1989) 5378–5383.
37. Hyrb D. J., Khan M. S. and Rosner W.: Testosterone-binding globulin binds to human prostate membranes. *Biochem. Biophys. Res. Commun.* **128** (1985) 432–440.
38. Fortunati N., Fissore F., Fazzari A., Berta L., Varvello L. and Frairia R.: Receptor for sex steroid-binding protein of endometrial membranes: solubilization, partial characterization, and role of estradiol in steroid-binding protein-soluble receptor interaction. *Steroids* **57** (1992) 464–470.
39. Hryb D. J., Khan M. S., Romas N. A. and Rosner W.: The control of the interaction of sex hormone-binding globulin with its receptor by steroid hormones. *J. Biol. Chem.* **265** (1990) 6048–6054.
40. Strel'chyonok O. A., Avvakumov G. V. and Survilo L. I.: A recognition system for sex-hormone-binding protein-estradiol complex in human decidual endometrium plasma membranes. *Biochim. Biophys. Acta* **802** (1984) 459–466.
41. Avvakumov G. V., Zhuk N. I. and Strel'chyonok O. A.: Subcellular distribution and selectivity of the protein-binding component of the recognition system for sex-hormone-binding protein-estradiol complex in human decidual endometrium. *Biochim. Biophys. Acta* **881** (1986) 489–498.
42. Krupenko N. I., Avvakumov G. V. and Strel'chyonok O. A.: Binding of human sex hormone-binding globulin–androgen complexes to the placental syncytiotrophoblast membrane. *Biochem. Biophys. Res. Commun.* **171** (1990) 1279–1283.
43. Fortunati N., Fissore F., Fazzari A., Berta L., Giudici M. and Frairia R.: Sex steroid-binding protein interacts with a specific receptor on human premenopausal endometrium membrane: modulating effect of estradiol. *Steroids* **56** (1991) 341–346.
44. Porto C. S., Abreu L. C., Gunsalus G. L. and Bardin C. W.: Binding of sex-hormone-binding globulin (SHBG) to testicular membranes and solubilized receptors. *Molec. Cell Endocr.* **89** (1992) 33–38.
45. Hansson V., Trygstad O., French F. S., McLean W. S., Smith A. A., Tindall D. J., Weddington S. C., Petrusz P., Nayfeh S. N. and Ritzen M.: Androgen transport and receptor mechanisms in the testis. *Nature* **25** (1974) 387–391.
46. Danzo B. J., Orgebin-Crist M. C. and Eller B. E.: Changes in 5α-dihydrostestosterone binding to epididymal cytosol during sexual maturation in rabbits: correlation with morphological changes in the testis and epididymis. *Molec. Cell Endocr.* **3** (1975) 203–220.
47. Frairia R., Fortunati N., Fissore F., Zeppegno P., Varvello L., Orsello M. and Berta L.: The membrane receptor for sex steroid binding protein is not ubiquitous. *J. Endocr. Invest.* **15** (1992) 617–620.
48. Wilson J. D.: Androgen abuse by athletes. *Endocrine Rev.* **9** (1988) 181–199.
49. Ashwell G. and Harford T.: Carbohydrate-specific receptors of the liver. *Ann. Rev. Biochem.* **51** (1982) 531–554.
50. Danzo B. J. and Eller B. C.: The ontogeny of androgen-binding protein in rat plasma, testis, and epididymis. *Endocrinology* **117** (1985) 1380–1388.
51. Rosenthal H. E.: A graphic method for the determination and presentation of binding parameters in a complex system. *Analyt. Biochem.* **20** (1967) 525–532.