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SEX STEROID BINDING PROTEIN (SBP) RECEPTORS IN ESTROGEN SENSITIVE TISSUES

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Summary—Since the discovery of a specific membrane binding site for sex steroid binding protein (SBP) in human decidual endometrium and in hyperplastic prostate numerous speculations have been raised on the existence of an additional non-receptor-mediated system for steroid hormone action. In the present work SBP cell membrane binding was investigated in human estrogen target tissues other than those previously studied either in the absence of steroids or in the presence of varying amounts (10^{-10}–10^{-6} M) of estradiol, testosterone and dihydrotestosterone, respectively. Plasma membranes obtained by differential centrifugation from homogenized samples of pre-menopausal endometrium, endometrium adenocarcinoma, normal liver and post-menopausal breast showed a specific binding of highly purified [^{125}I]SBP: a major displacement of labeled SBP was elicited by radioinert SBP, while no significant displacement occurred when other human plasma proteins were used as cold competitors (molar excess ranging 500–10,000-fold). A specific, time-dependent binding of [^{125}I]SBP was also observed in MCF-7 and in Hep-G2 cell lines. The different patterns of specific binding, observed in membranes from different tissues when SBP was liganded with different sex steroid molecules, leads us to consider the tissue individuality of the receptor as a further entity in the membrane recognition system for SBP.

INTRODUCTION

In human plasma, sex steroid hormones are mostly bound to sex steroid binding protein (SBP) and only a small fraction is unbound.

Until recently, general consensus has supported a role for SBP as a simple buffer reservoir for the most important naturally occurring sex steroids: testosterone, dihydrotestosterone and estradiol. The immunocytochemical detection of intracellular SBP [1–4] in extravascular sites (other than its place of synthesis) together with evidence that protein bound steroids may leave the vascular compartment, have introduced a modification of the traditional view.

In the last few years, a number of observations have been raised concerning the existence of a specific membrane binding site for SBP in some human steroid target tissues: prostate [5], decidual endometrium [6] as well as solubilized receptor from prostatic membranes [7]. Even if sophisticated biochemical data have been obtained on SBP–receptor interaction, the physiological role of such a system is still unclear.

The bulk of information has been enriched by data about the influence of sex steroids on SBP-membrane binding. Presently, two opposite models have been proposed.

Rosner [8] suggested an inhibitory activity exerted by sex steroids, when SBP binds the hormone before interacting with prostate-membrane receptor. On the contrary, SBP complexed with its receptor retains its full steroid binding activity. In addition, the author reported a significant increase of intracellular cAMP on LNCaP cells following SBP–receptor interaction.

Strel'chyonok and Avvakumov [9] proposed a model obtained on decidual endometrium. Their data support the view that membrane receptor interacts only with SBP–estradiol complex, while no interaction occurs either with unliganded SBP or with SBP–androgen complex. The authors suggested a shuttle mechanism, in which, after the interaction of SBP–estradiol complex with membranes, only estradiol is internalized into target tissue.

The aim of the present study was to investigate whether SBP-membrane binding is present in estrogen target tissues other than the above cited, as well as in some cultured cells and how sex steroids can affect the function of the system. Awareness of either the difference or

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similarity, if any, of the behavior of SBP receptor in different target cells could offer an effective grounding to the understanding of its physiological role.

EXPERIMENTAL

Tissue samples

Data in the present study were obtained in the following human tissues: pre-menopausal endometrium, endometrium adenocarcinoma, normal liver and post-menopausal breast.

All samples were obtained during surgery for major clinical problems, such as vaginal hemorrage either for uterine fibroma or suspicion of neoplasm, diagnostic biopsy or suspicion of mammary neoplasia (not histologically confirmed).

Plasma membranes were obtained following the differential centrifugation technique as described by Singer *et al.* [10], after homogenization in 25 mM Tris–HCl, 0.3 M sucrose, pH 7.4. Protein content [11], as well as 5′-nucleotidase activity (marker of plasma membranes [12]) were measured both in homogenates and in membrane suspensions.

Binding experiments were performed using pure human SBP labeled with ^{125}I (10 mCi/mg) purchased from Milab (Sweden). Human radioinert highly purified SBP was kindly provided by P. H. Petra, University of Washington, Seattle, U.S.A. Human albumin, transferrin, orosomucoid and steroid hormones (estradiol, testosterone and dihydrotestosterone) were obtained from Sigma Chemicals (St Louis, MO, U.S.A.). Asialo-orosomucoid was prepared with agarose bound neuraminidase (type X) from *Clostridium perfringens* (Sigma Chemicals) according to the instructions of the manufacturer.

Binding took place in plastic gamma-counter tubes pre-treated with 25 mM Tris–HCl, 10 mM $CaCl_2$, 0.2% BSA, pH 7.4 (binding buffer, BB) for 1 h at 37°C.

Aliquots of membranes (50–100 μg protein/tube) were incubated with selected quantities of [^{125}I]SBP, either in the absence or in the presence of a 1000-fold molar excess of radioinert SBP to account for non-specific binding, for 24 h at 4°C with continuous rotation. At completion, tubes were centrifuged at 2000*g* for 15 min. at 4°C to pellet membranes. Supernatants were discarded and pellets counted for radioactivity. Binding specificity was evaluated using as cold competitors radioinert SBP as well as other human plasma proteins in molar excess ranging 500–10,000-fold.

The effects of sex steroids on [^{125}I]SBP membrane binding was studied by adding radioinert testosterone (Te), estradiol (E_2) and dihydrotestosterone (DHT) in concentrations ranging 10^{-10}–10^{-6} M. Standard incubation was preceded by a pre-incubation for steroids and SBP (labeled and radioinert) of 15 min at room temperature with gentle shaking to allow the hormones and their carrier protein to equilibrate.

On pre-menopausal endometrium membrane only an adjunctive experimental condition was scheduled. Aliquots of membranes (about 100 μg protein/tube) were incubated at 4°C with: (a) [^3H]DHT (60 Ci/mmol; Amersham, U.K.) 10^{-9} M for 24 h; (b) radioinert SBP 10^{-9} M plus [^3H]DHT 10^{-9} M, pre-incubated for 1 h at 4°C, for 23 h; (c) radioinert SBP 10^{-9} M for 20 h at 4°C and with a subsequent addition of [^3H]DHT 10^{-9} M for 4 h.

All determinations were done in duplicate. Binding parameters are expressed in the text as mean $\pm$ SD, calculated from a minimum of three experiments.

Cell culture

Two different cell lines were also taken into account: MCF-7, an estrogen sensitive human mammary carcinoma cell line [13] donated to us by L. Cesano, University of Torino; Hep-G2, a human hepatoma cell line [14], donated to us by G. C. Actis, Ospedale Molinette, Torino.

Cells were maintained at 37°C in RPMI 1640 containing 10% heat inactivated foetal calf serum. About ten days before binding experiments, sub-confluent cells were exposed to trypsin (0.05%)–EDTA (0.02%) solution (GIBCO, Paisley, U.K.) for 5–10 min. The collected cells were seeded in 25 cm^2 flasks (Falcon, NJ, U.S.A.) in the previously described medium and maintained at 37°C. When a good adhesion state was obtained, cells were switched for 48–72 h to serum-free medium (RPMI 1640 supplemented with 0.5% insulin, 0.5% transferrin, 0.05% sodium selenite and 0.5% BSA (Sigma, St Louis, MO, U.S.A.). At 90–95% confluence (3.0–3.5 $\times$ 10^6 cells/flask for MCF-7; 4.5–5.0 $\times$ 10^6 cells/flask for Hep-G2) cells were used for experiments.

Prior to binding, cells were washed three times with sterile phosphate-buffered saline (PBS) at 4°C. To initiate binding, 5 ml of RPMI

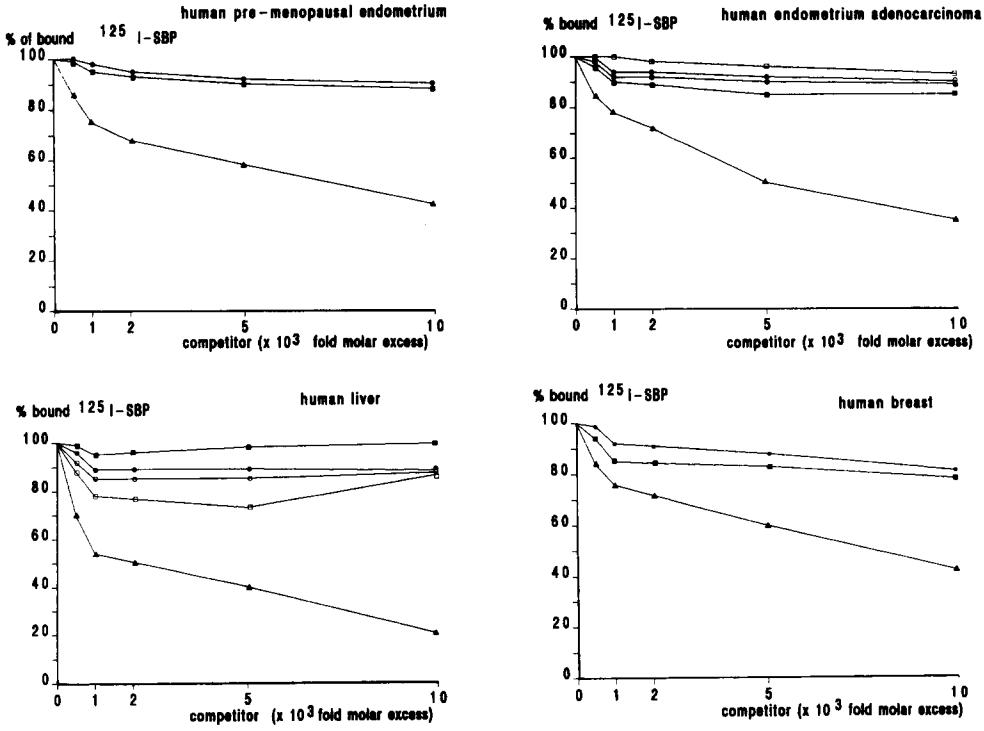

Fig. 1. Competition to [^{125}I]SBP-membrane binding exerted in four different human tissues by the following human radioinert plasma proteins: SBP (▲—▲); albumin (●—●); transferrin (■—■); orosomucoid (○—○); and asialo-orosomucoid (□—□). [^{125}I]SBP = 10^{-9} M; radioinert competitors = 500–10,000-fold molar excess; [^{125}I]SBP binding in the absence of radioinert competitor = 100%

1640 supplemented with 0.75% BSA maintained at room temperature was added to each flask along with a selected quantity of [^{125}I]SBP, either in the absence or in the presence of 1000-fold molar excess radioinert SBP to account for non-specific binding. After incubation at room temperature on a rocker table for the appropriate time, media were removed and flasks were washed three times in PBS at 4°C. To obtain cell lysates, 1–2 ml of 2 N NaOH were added to each flask for 2–3 min. Lysates were, then, removed and counted for radioactivity.

All determinations were done in duplicate and each experiment was repeated at least twice.

RESULTS

Tissue samples

Pre-menopausal endometrium, endometrium adenocarcinoma, normal liver and post-menopausal breast have been demonstrated to present a specific membrane binding for [^{125}I]SBP. Radioinert SBP competed with labeled protein, whereas other plasma proteins did not exert any significant displacement (Fig. 1). The binding of [^{125}I]SBP to pre-menopausal endometrium has also been shown to be saturable and at high affinity [15]. In addition it seems to occur at two binding sites at different affinity as described for decidual endometrium and prostate. The same binding characteristics have also been proved in endometrium adenocarcinoma (Fig. 2).

Sex steroids seem to affect SBP-membrane interaction in an intricate fashion. When SBP complexed with steroids, used at concentrations close to physiological values (10^{-9}–10^{-8} M), was incubated with membranes, either an enhancement or an inhibition of binding was found (Fig. 3). Our data appear to support a tissue specific response to an individual type of steroid molecule. In liver and endometrium adenocarcinoma only testosterone demonstrates an enhancing effect; estradiol acts in the same way on pre-menopausal endometrium, while in post-menopausal breast all three steroids show an inhibiting effect. However, at steroid concentrations above 10^{-8} M, estradiol, testosterone and DHT have been shown to inhibit at a significant extent SBP-membrane binding in every tissue under study (Fig. 4).

A comparable result has been obtained when pre-menopausal endometrium membranes were incubated with radioinert SBP complexed with [^{3}H]DHT (10^{-9} M). The radioactivity estimated

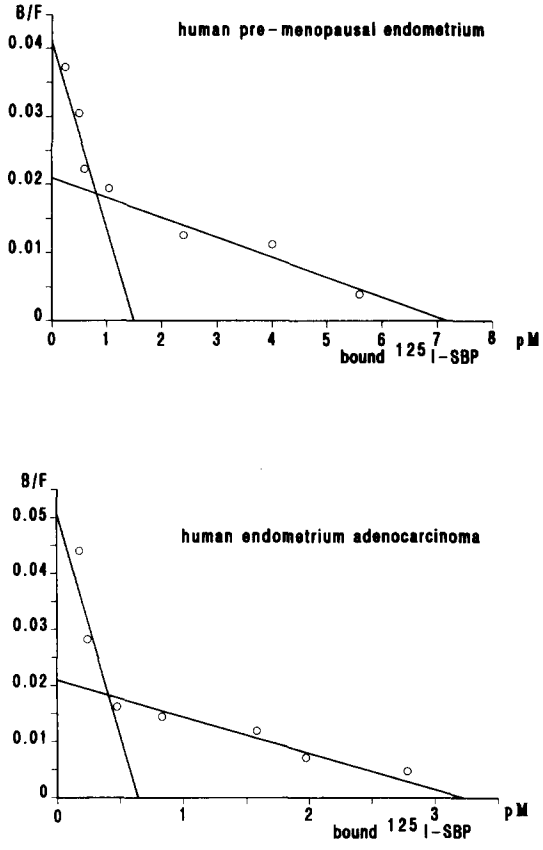

Fig. 2. Scatchard plots of [^{125}I]SBP binding to plasma membranes of either human pre-menopausal endometrium and human endometrium adenocarcinoma. Pre-menopausal endometrium: left branch, $K_d = 3.6 \pm 1.1 \times 10^{-11}$ M, $B_{max} = 18.8 \pm 4.5$ fmol of ^{125}I-SBP/mg membrane protein; right branch, $K_d = 3.4 \pm 1.8 \times 10^{-10}$ M, $B_{max} = 91.4 \pm 12.3$ fmol of [^{125}I]SBP/mg membrane protein. Endometrium adenocarcinoma: left branch, $K_d = 1.2 \pm 0.4 \times 10^{-11}$ M, $B_{max} = 6.5 \pm 2.8$ fmol of [^{125}I]SBP/mg membrane protein; right branch, $K_d = 1.5 \pm 0.6 \times 10^{-10}$ M, $B_{max} = 32.2 \pm 6.2$ fmol of [^{125}I]SBP/mg membrane protein.

in the pellet was not different from that recovered after incubation of plasma membranes and [^{3}H]DHT alone. On the contrary, when [^{3}H]DHT was added to radionert SBP–plasma membrane complex, a significant increase of pelleted radioactivity was observed. Experimental procedure and results are shown in Fig. 5.

Cell culture

[^{125}I]SBP was demonstrated to bind specifically to MCF-7 and Hep-G2 cells. In both cell lines, the binding was shown to be time-dependent (Fig. 6).

DISCUSSION

Since the immunohistochemical detection of a plasma SBP-like binding protein in the cytoplasm of endocrine target cells [1–4], a great number of experiments have been devised in order to explore the biological role and importance of intracellular steroid binding proteins other than "classic" receptors. With the recent demonstration of a specific binding of SBP to plasma membranes of human prostate and decidual endometrium [5–9, 16], a recognition system furnishing a further mechanism for steroid entry into cells was hypothesized [9]. The hypothesis was based on the observation that specific binding of SBP to membranes from decidual endometrium was evidenced only in the presence of estradiol. Nevertheless, when dihydrotestosterone was used as the ligand for SBP, the specific binding of the glycoprotein to plasma membranes was completely abolished. Most recent data from Hryb *et al.* [17] are at variance with the speculated biological steroid specificity of the SBP–receptor interaction. The authors suggest that unliganded SBP is able to bind either steroids or membrane-receptor in a reversible reaction; liganded SBP cannot bind to its receptor, but unliganded SBP, that first binds to receptor, can subsequently bind steroids.

The first aim of our work was to elucidate whether specific membrane binding for SBP was demonstrable in some other estrogen sensitive tissues. Furthermore, we were interested in estimating estrogen or androgen effects on SBP-membrane binding in different target tissues. The evaluation of target tissues in different physiological and pathological conditions could provide further data on the role and function control of SBP–receptor interaction.

On the basis of our data, unliganded SBP specifically binds to membranes obtained from pre-menopausal endometrium, endometrium adenocarcinoma, normal liver and post-menopausal breast. A further characterization of the binding in both endometrium samples demonstrate all the features of receptor binding. K_d obtained for the high affinity sites, in pre-menopausal endometrium and in endometrium adenocarcinoma, are in the range of receptor affinity constant (pre-menopausal endometrium: $K_d = (3.6 \pm 1.1) \times 10^{-11}$ M; endometrium adenocarcinoma: $K_d = (1.2 \pm 0.8) \times 10^{-11}$ M). The existence of an SBP-membrane receptor in pre-menopausal endometrium as well as in post-menopausal endometrium neoplasia allows us to assume that the receptor is not the expression of estrogen over-stimulation on their target tissues. Either decidual endometrium or hyperplastic prostate, where the

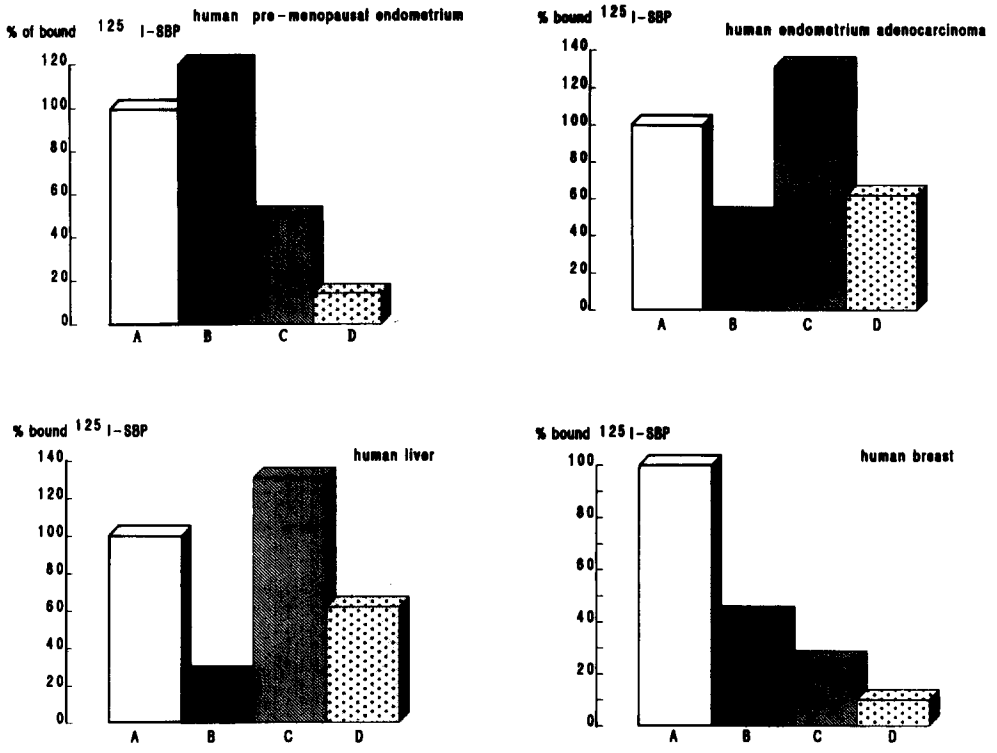

Fig. 3. Specific binding of [^{125}I]SBP to plasma membranes of four different human tissues, either in the absence or in the presence of steroid hormones ("single point" experiments). (A) in the absence of sex steroids (specific binding = 100%); (B) in the presence of 10^{-8} M estradiol; (C) in the presence of 10^{-9} M testosterone; (D) in the presence of 10^{-9} M dihydrotestosterone.

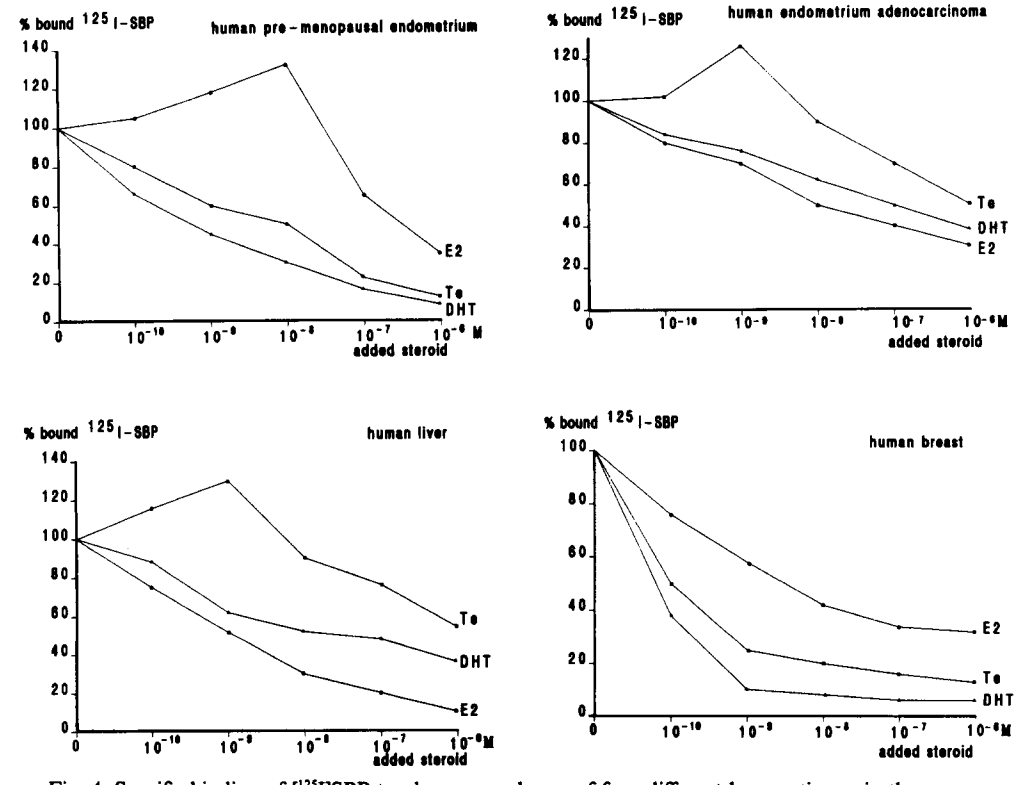

Fig. 4. Specific binding of [^{125}I]SBP to plasma membrane of four different human tissues in the presence of sex steroid hormones (E_2, Te, DHT), in concentration ranging 10^{-10}–10^{-6} M. Specific binding in the absence of steroids = 100%.

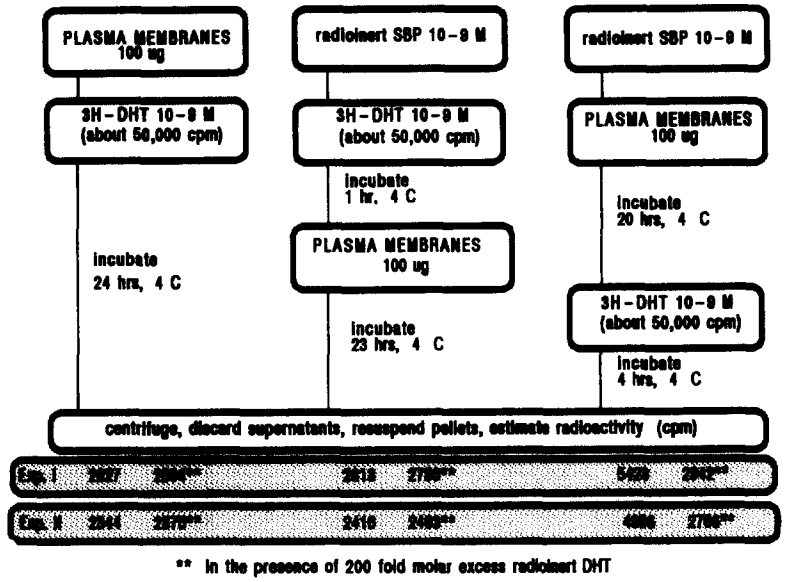

Fig. 5. Binding of [^{3}H]DHT to plasma membranes of human pre-menopausal endometrium, either in the absence or in the presence of equimolar concentration of radioinert SBP (10^{-9} M); (see the text).

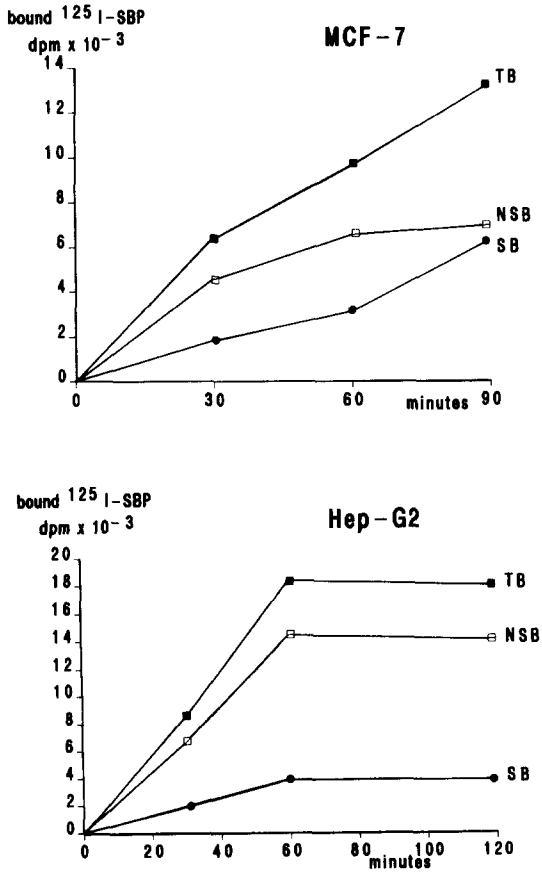

Fig. 6. Time-course (25°C) binding of [^{125}I]SBP to MCF-7 and Hep-G2 cells. TB = total binding, in the absence of radioinert SBP. NSB = non-specific binding, in the presence of 1000-fold molar excess of radioinert SBP. SB = specific binding, calculated by subtracting NSB from TB.

receptor had been previously described, are known to be the morphological expression of estrogen and/or androgen over-stimulation on tissue in basal conditions.

Our preliminary detection of a specific binding for SBP also on membranes from post-menopausal breast, even if not yet fully characterized, supports the view that the SBP-membrane receptor is a physiological component of estrogen sensitive tissues.

For the first time, liver, which has to be considered not only as sex steroid sensitive tissue, but also as a crucial tissue in SBP metabolism, has been shown in the present study to display a specific membrane binding for the protein.

Our main concern in evaluating liver receptor was to exclude a possible binding of [^{125}I]SBP to the well known asialo-glycoprotein receptor. Liver membranes, in fact, have been described to present a specific receptor for asialo-glycoproteins [18]. Procedures adopted to purify and label human SBP cannot be excluded to cause some modification of glycidic structure of the protein. In this case a binding to asialo-glycoprotein receptor could occur. The observed lack of significant displacement exerted by asialo-orosomucoid on [^{125}I]SBP bound to liver membranes has to be stressed.

Results obtained with both MCF-7 and Hep-G2 cells suggest that these cell types should also present a specific receptor for SBP. Data are still scanty and totally preliminary, but, at least in

MCF-7 cells, our finding together with previously described intracellular SBP in the same cell line [1] prompts detailed studies in the future. In addition, the study of other mammary carcinoma cell lines lacking estrogen receptor may provide further information on SBP-membrane receptor and estrogen-cell receptor interrelationship.

Data obtained in our laboratory suggest that also SBP complexed with sex steroids can bind to its membrane receptor, but only in very distinctive conditions. First of all, each different tissue seems to act in a sort of individual manner. Post-menopausal breast membranes, for example, do not bind complexed SBP, either with estradiol or with androgens. Pre-menopausal endometrium membranes, on the other hand, have been shown to bind SBP–estradiol complex (estradiol 10^{-9}–10^{-8} M), while androgens act as inhibitors of SBP-membrane interaction. Yet, another behavior has been evidenced in liver as well as in endometrium adenocarcinoma membranes. In these last cases, the binding occurs for SBP complexed with testosterone (10^{-9} M), but not with either DHT or estradiol. It has to be noticed that these data are true only for steroid concentrations close to physiological range. When steroids, either estradiol or testosterone or DHT, had been used at concentrations over 10^{-8} M, only an increasing inhibition of the binding of SBP–steroid complex to membranes occurs, despite the nature of tissue.

As far as pre-menopausal endometrium membranes are concerned, the observation that while DHT bound to SBP inhibits the protein interaction with membranes, membrane bound SBP is still able to bind DHT is remarkable. Our results, in this case, completely agree with evidence provided by Hryb *et al.* [17] on receptor solubilized from prostate membranes.

The present knowledge has not yet furnished a univocal opinion about the physiological significance of the SBP-membrane receptor system. Actually, two different explanations have been proposed. Strel'chyonok and Avvakumov [9] consider the SBP estadiol-membrane receptor system as an additional mechanism of uptake of estradiol into cells. In their opinion, the internalization occurs only for the hormone which is recognized by membrane receptor as a complex with SBP. Such a mechanism, called receptor-mediated endocytosis, has been described to occur in several cell types for the uptake of biologically important molecules [19], like for the uptake of low density lipoprotein-bound cholesterol [20].

Rosner [8], rejecting a specific binding of the pre-formed steroid–SBP complex to membranes, suggests indeed an interaction between receptor membrane and SBP that can be only subsequently liganded by steroids. The response of cells to the complete system interaction is suggested to result in an activation of cell second messenger, cAMP.

Nevertheless, experimental data available to date do not seem sufficient to provide conclusive evidence for the validity of either one or the other hypothesis.

The present study is not conclusive as far as the function of SBP–receptor system is concerned. However, our findings suggest that the membrane recognition system for SBP (or steroid-complexed SBP?), which appears to give rise to certain effects typical for protein hormones not penetrating through cell membranes, has to be enriched by a further entity: tissue individuality of receptor. Different target tissues, as well as different physiological or pathological conditions, and possible pharmacological manipulations have to be considered as factors able to modulate SBP-membrane receptor interaction. Target cells could play the role of selective amplifier of steroid action through membrane receptor for SBP which can evoke transmembrane transfer of hormonal signal.

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