Pergamon

0960-0760(94)00156-1

J. Steroid Biochem. Molec. Biol. Vol. 52, No. 2, pp. 173–179, 1995

0960-0760/95 $9.50+0.00

Over-expression of Human Sex Steroid-binding Protein (hSBP/hABP or hSHBG) in Insect Cells Infected with a Recombinant Baculovirus. Characterization of the Recombinant Protein and Comparison to the Plasma Protein

Li-ming Sui, Cynthia Wong and Philip H. Petra*

Department of Biochemistry, University of Washington, Seattle, WA 98195, U.S.A.

Human sex steroid-binding protein (hSBP/hABP or hSHBG) was over-expressed in High Five and Sf9 cells adhered to plates and in suspension. The adherent cells expressed to levels of 2.3 mg/l and 1.4 mg/l after 4 and 6 days, respectively, while Sf9 cells grown in suspension yielded 4.67 mg/l after 6 days. Recombinant hSBP/hABP, purified to homogeneity by immunoadsorption, was found to fold similarly to native plasma hSBP/hABP and to display similar sequence epitopes after heat denaturation. The recombinant protein binds dihydrotestosterone, testosterone, and 17β-estradiol with K_ds of 0.6, 2.4, and 14.2 nM, respectively, which are similar to plasma hSBP/hABP. The recombinant protein contains N-linked and O-linked oligosaccharide side-chains but the monomer exhibits a slightly lower molecular weight than plasma hSBP/hABP (40 kDa vs 44 kDa) which may be due to the absence of one N-linked side-chain or to shorter oligosaccharide side-chains. The partial N-terminal sequence LRPVLP(T)Q of recombinant hSBP/hABP is identical to plasma hSBP/hABP but appears to be less heterogeneous. These results indicate that recombinant baculovirus SBP represents a good model for investigating the structure of plasma hSBP/hABP. The expression system will allow the isolation of preparative amounts of SBP mutants generated by combinatorial site-directed mutagenesis to advance investigations on structure–function relationships and undertake crystallization trials for X-ray diffraction analyses.

J. Steroid Biochem. Molec. Biol., Vol. 52, No. 2, pp. 173–179, 1995

INTRODUCTION

Human sex steroid-binding protein, hSBP/hABP (or human sex hormone binding globulin, hSHBG) is a 93.4 kDa homodimeric glycoprotein that circulates in the plasma of many species, including humans, and specifically binds 1 mole of dihydrotestosterone (DHT), testosterone (T), and 17β-estradiol (E_2) per dimer with K_ds of about 0.3, 1, and 4 nM, respectively [1, 2]. SBP appears to have dual roles in plasma, one to control the metabolic clearance rates of T and E_2 [3, 4], and the other to assist in their diffusion into target cells through the participation of an SBP membrane receptor [5–8]. SBP is present in the cytoplasm of tissues that respond to sex steroid hormones [9, 10] and is taken up by MCF-7 cells [9], a human breast cancer cell line, supporting its proposed role in sex steroid hormone uptake. The amino acid sequence of hSBP [11] is identical to that of human testis androgen-binding protein, hABP, as deduced from cDNA sequencing [12, 13] indicating that both proteins are encoded by the same gene. The deduced protein sequence of rat ABP, however, is only 68% identical to hSBP/hABP [14] with a K_d (DHT) 10-fold higher than hSBP/hABP (2.5 nM

*Correspondence to P. H. Petra.

Abbreviations: SBP, plasma sex steroid-binding protein; SHBG, sex hormone binding globulin; ABP, testicular androgen binding protein; hSBP, human SBP; hABP, human ABP; rABP, rat testicular androgen binding protein; BacSBP, SBP expressed in recombinant baculovirus-infected cells; DHT, 5α-dihydrotestosterone; T, testosterone; E_2, 17β-estradiol; P, progesterone; F, cortisol; SDS, sodium dodecyl sulfate; GnHCl, guanidine hydrochloride; Con A, Concanavalin A from *Canavalia ensiformis*; WGA, wheat germ agglutinin from *Triticum vulgaris*; PNA, peanut lectin from *Arachis hypogaea*; Tween 20, polyoxyethylenesorbitan; PBS, phosphate buffered saline; NFM, Carnation nonfat dry milk powder; BSA, bovine serum albumin.

Received 3 Aug. 1994; accepted 22 Sep. 1994.

[15] vs 0.25 nM [1, 16, 17]) strongly suggesting that the steroid-binding site as well as other regions of the rat protein are different from hSBP/hABP. The low expression level of hSBP/hABP in transfected BHK-21 [18], COS-7 cells [19]* and CHO cells [20] has precluded biochemical characterization of the recombinant wild type protein but has nonetheless permitted limited interpretation of site-directed mutagenesis experiments [19]. In this paper, we describe the over-expression of SBP in the baculovirus expression system, reported to be one of the most efficient for expressing eucaryotic proteins in their glycosylated forms [21], and the characterization of the recombinant SBP and its comparison to SBP isolated from human serum.

MATERIALS AND METHODS

Materials

The sources of materials were: human pregnancy serum (Dr Katheryn Moore at Madigan Army Hospital, Tacoma WA); Sf9 cells, High Five cells, pcDNAIneo, pBlueBacIII, linear AcMNpV viral DNA, and cationic liposome solution (Invitrogen, San Diego, CA, U.S.A.); Hinks's TNM-FH and Ex-Cell 400™ media (JRH Biosciences, Lenexa, KS, U.S.A.); fetal calf serum and SF-900II serum-free medium (Gibco/BRL, Grand Island, NY, U.S.A.), succinimidyl-6-(biotinamido)hexanoate (# 21335) (Pierce Chemicals, Rockfort, IL, U.S.A.), streptavidin, biotinylated alkaline phosphatase, and rabbit anti-goat IgG coupled to alkaline phosphatase (Bio-Rad, Hercules, CA, U.S.A.); biotinylated Con A, WGA, PNA, and avidin-alkaline phosphatase (E.Y Laboratories, San Mateo, CA, U.S.A.); and nitrocellulose membranes (cat# HAHY 304FO) (Millipore, Bedford, MA, U.S.A.). All other chemicals used were reagent grade as described in previous publications from this laboratory.

DNA constructions, cell culture, and transfections

Sf9 and High Five cells were grown either in monolayer or suspension in Hinks's TNM-FH and Ex-Cell 400™ media, respectively. For Sf9 cells, media were supplemented with 10% fetal calf serum and replaced by Sf-900II serum-free medium after viral infection. Human SBP cDNA cloned in pBluescript II [18] was subcloned into BamH1 and HindIII sites of transfer vector pBlueBacIII. 2×10^6 Sf9 cells in a 60 mm plate were co-transfected with 3 μg of pBlueBacIII/SBP and 1 μg of linear AcMNpV viral DNA using cationic liposome according to instructions from Invitrogen. Several recombinant virus plaques were selected by ELISA and [^{3}H]DHT binding [19] and recombinant virus was purified by six rounds of plaquing, picking a single blue plaque at each round. An expression time-course was established by infecting monolayer cultures of 2×10^6 of Sf9 and High Five cells with a pure stock of recombinant virus having a titer of 10^6 plaque forming unit (pfu) to make a multiplicity of infection (MOI) greater than 2.

Purification of BacSBP and plasma SBP

Infected insect cell medium was concentrated 10-fold by ultrafiltration (YM-10 Amicon membrane) and BacSBP was purified by immunoadsorption on a column (0.9 cm×7 cm) of polyclonal antiSBP-agarose equilibrated in 100 mM NaCl–50 mM Tris–Cl, pH 7.5 [9]. Plasma hSBP was purified from pregnancy serum as previously described [9, 22] with modifications [23]. SBP concentration was determined spectrophotometrically using $\epsilon_{280}=1.14\times10^5$ cm^{-1} M^{-1} [24].

Electrophoresis and blotting methods

Proteins electrophoresed in SDS slabs [24] were transferred to nitrocellulose membranes with the Transblot apparatus according to Bio-Rad instructions, fixed, and probed with goat anti-SBP and rabbit anti-goat IgG coupled to alkaline phosphatase [18, 25]. The presence of oligosaccharide side-chains was determined by probing the transferred membranes with biotinylated Con A, WGA, and PNA followed by avidin-alkaline phosphatase. Polyclonal monospecific hSBP antibodies were affinity-purified from goat plasma by passing through a column of protein G-agarose followed by a column of hSBP-agarose [9].

Competitive strepavidin-biotin ELISA

Biotinylated hSBP was prepared by reacting 550 μg succinimidyl-6-(biotinamido)hexanoate with 600 μg pure hSBP (15:1 mol ratio) in 1 ml 50 mM bicarbonate, pH 8.5, for 2 h at 0°C. The reaction was stopped with 10 mM Tris–Cl, pH 7.4. Unbound reagent was removed by centrifuging in Centricon-10, 2 ml three times. The ELISA was carried out as follows: wells of a microtiter plate were coated overnight at 4°C with 100 μl affinity-purified polyclonal anti-hSBP (2.8 mg/ml). The plate was emptied into a sink, patted dry, and washed three times with 0.5% (w/v) Tween 20 in PBS (8 g NaCl, 0.2 g KCl, 0.61 g Na_2HPO_4, 0.2 g HK_2PO_4/l). Wells were blocked with 200 μl 5% NFM for 1 h at 25°C and the plate was emptied into a sink and patted dry. 0.1 nM Biotinylated SBP (50 μl) mixed with either 50 μl PBS or 50 μl containing increasing concentrations of wild type hSBP, BacSBP, heat-denatured (60°C, 1 h) hSBP or BacSBP, were added and incubated at 37°C for 1 h. The plate was emptied into a sink and patted dry. Streptavidin was diluted 1:1500 with PBS containing 0.05% Tween-0.25%BSA–0.1% NaN_3, and 100 μl were added to each well and incubated 1 h at 25°C. Biotinylated alkaline phosphatase was diluted 1:125 with PBS containing 0.05% Tween-0.25% BSA–0.1% NaN_3 and 50 μl were added to wells (in the presence of streptavidin), mixed, and incubated for 1 h at 25°C. The

*Presence of 5% calf serum in COS-7 expression media required a small correction in the DHT binding activity of mutants due to the presence of bovine SBP. Transfections repeated in serum-free media gave results identical to the corrected ones reported in Ref. [19].

plate was emptied into a sink, patted dry, washed 3 times with 0.5% (w/v) Tween 20 in PBS, 3 times with 10 mM diethanolamine–0.5 mM $MgCl_2$ (pH 9.5), emptied and patted dry. *p*-Nitrophenyl phosphate (100 μl, 2 mg/ml) was added to each well and reaction was stopped after 20 min with 50 μl 1 N NaOH. The plates were read at 405 nm.

Steroid-binding analyses

Samples from baculovirus-infected cell media were collected daily for 12 days to measure DHT binding activity by charcoal adsorption [19]. K_ds of DHT, T, and E_2 were determined by competitive Scatchard analyses [26] at 4°C, pH 7.4, on media diluted to 1 nM BacSBP. The constants were calculated from: $K_p=K_d(1+[I]/K_i)$, where K_d is the equilibrium constant of [^{3}H]DHT dissociation, K_p is K_d in the presence of [I] competitor (6.67 nM of radioinert T or E_2), and K_i is the equilibrium constant of T or E_2 dissociation. Competition with progesterone and cortisol was carried out as published [18].

Automated Edman degradation

BacSBP was blotted on PVDF membranes and sequenced directly with an Applied Biosystems model 470 Sequencer with on-line phenylthiohydantoin analysis using published programs protein [27].

RESULTS

Figure 1 shows the time-course of BacSBP expression in adherent Sf9 and High Five cells. The former cells yield 15 nM BacSBP (1.4 mg/l) after 6 days of infection. The DHT binding activity and total expressed SBP remain constant for at least 12 days after infection as measured by immunoblots (lanes 3–5). The High Five cells yield about 25 nM BacSBP (2.3 mg/l) after 4 days but the activity falls to 7 nM after 10 days and the expressed protein is no longer detectable on immunoblots (lanes 6 and 7). Although the monomeric structure of SBP, and presumably its dimeric structure, remain unchanged in the early stages of expression as

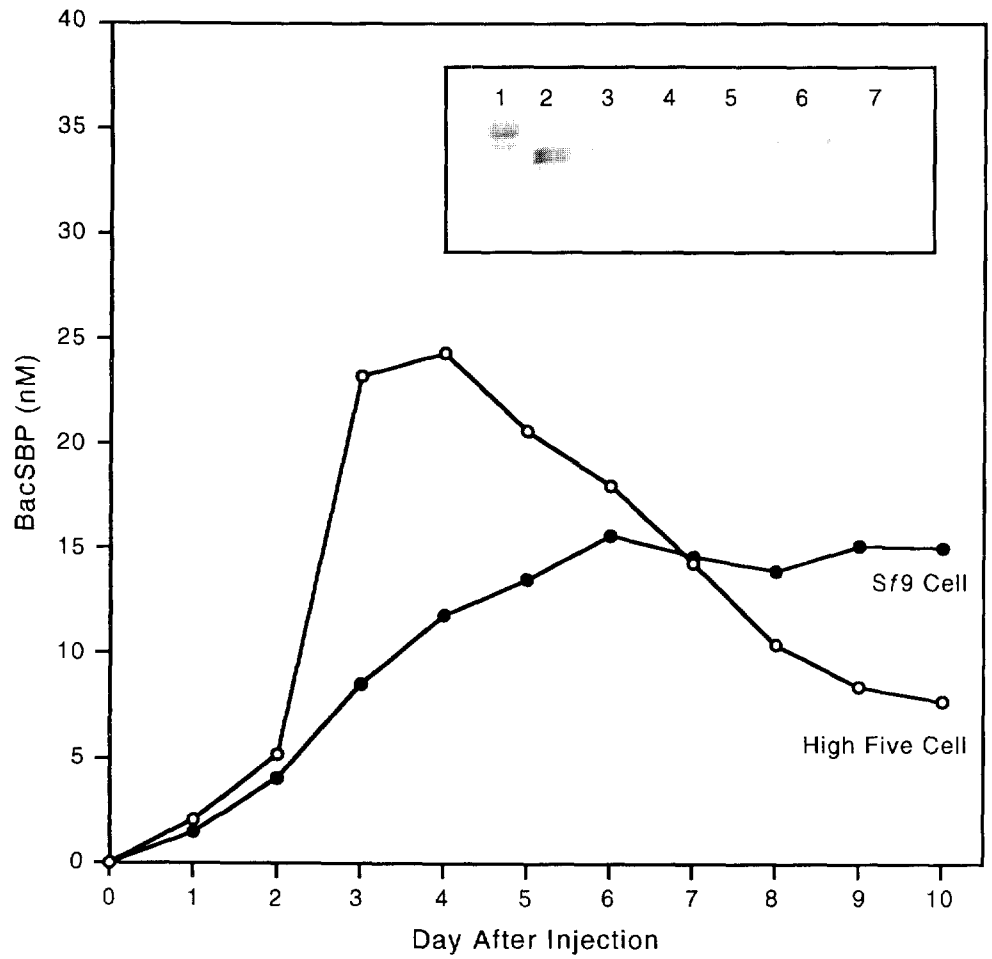

Fig. 1. SBP expression time-course. Cells were grown as described in Methods. BacSBP concentration was calculated from [^{3}H]DHT binding activity [19]. Immunoblots in the inset are: lane 1, 2.5 μg control plasma hSBP/hABP; lane 2, 2.5 μg control BacSBP purified from infected Sf9 cell medium; lanes 3–5, 25 μl aliquots of Sf9 cell 15× concentrated medium (1 ml total) from day 6, 10, and 12; lanes 6 and 7, 25 μl aliquots of infected High Five cell 15× concentrated medium (1 ml total) from day 4 and 10, respectively.

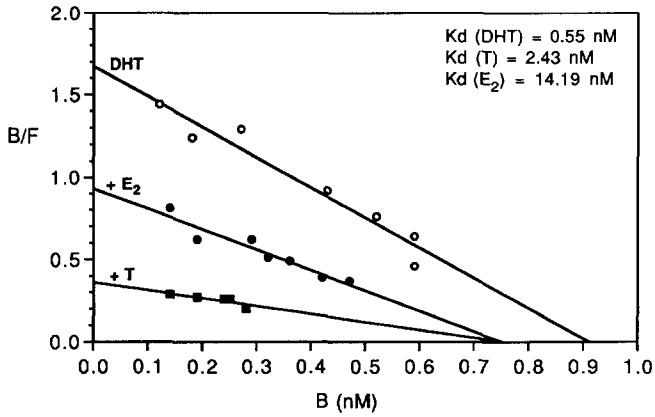

Fig. 2. Determination of equilibrium constants of DHT, T, and E_2 dissociation from BacSBP expressed in Sf9 cells by competitive Scatchard analysis as described in Methods.

indicated in the immunoblots, the decline of steroid-binding activity after 4 days coincides with the disappearance of the BacSBP band (lane 7) and the appearance of low molecular weight fragments indicating cleavage by proteases (data not shown). Expression levels of 50 nM BacSBP (4.67 mg/l) were obtained with Sf9 cells grown in suspension in 1 l spinner flasks for 6 days, and we expected to attain expression levels of at least 100 nM (9.3 mg/l) in 4 days with High five cells grown in suspension (preliminary data). As shown in the competitive Scatchard plot (Fig. 2), BacSBP binds DHT and T with K_ds of 0.55 nM and 2.43 nM at 4°C which are similar to those of hSBP/hABP within experimental error (0.42 and 1.89 nM) [26]. The higher K_d value of 14.19 nM for E_2 (compared to 4.79 nM for hSBP/hABP [26] probably results from slight errors in the concentration of radioinert E_2 used in the competitive Scatchard analysis. BacSBP does not bind progesterone and cortisol (data not shown) as previously found for hSBP/hABP and SBP expressed in BHK-21 [18].

The molecular properties of BacSBP are shown in Fig. 3. The molecular weight of the BacSBP subunit is about 40 kDa as determined by coomassie blue staining [Fig. 3(E, lane 1)] and by immunoblotting [Fig. 3(D, lane 1)]. The value is slightly lower than plasma hSBP/hABP (44 kDa from Fig. 3(E, lane 2 and D, lane 2)]. The immunoblot of Fig. 3(D) also reveals three distinct BacSBP monomers (lane 1) compared to only two for hSBP/hABP in this particular preparation (lane 2). Presence of *β-N*-acetyl-glucosamine and branched mannoses of the putative N-linked side-chains were shown by probing the transferred cellulose membranes with Con A [Fig. 3(C)] and WGA [Fig. 3(B)]. Presence of galactose and *N*-acetylgalactosamine in the O-linked side-chain on Thr^7 was shown by probing with *Artocarpus integrifolia* (Jacalin) [Fig. 3(A)]. All these lectins were found to bind to human SBP [25]. The lower molecular weight monomer of BacSBP, shown as the lower band in lane 1 of Fig. 3(D), does not appear to be glycosylated since the corresponding band is not present in the Con A and WGA lectin blots.

The conformation of BacSBP was compared to that of the native human plasma protein by using the amplified competitive streptavidin–biotin ELISA shown in Fig. 4. The data indicate that BacSBP and human plasma

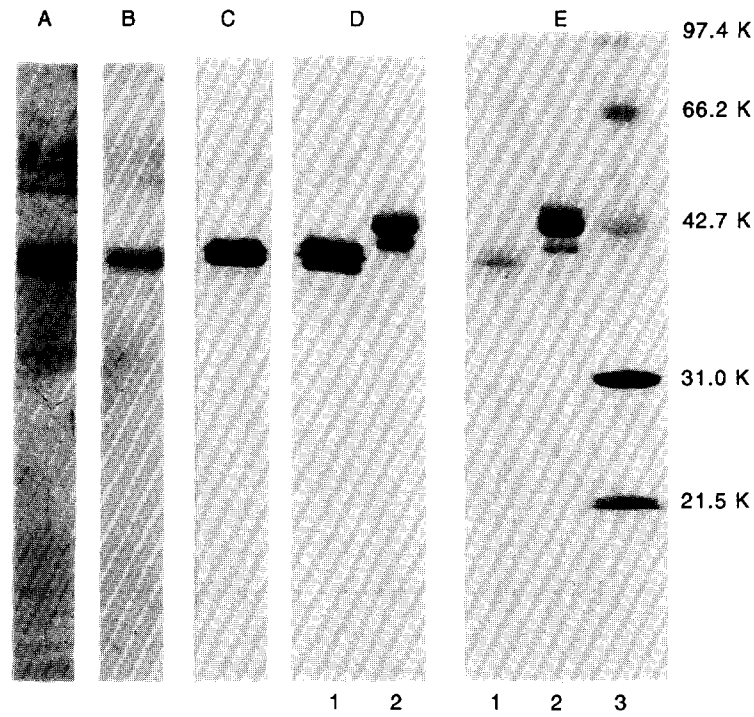

Fig. 3. SDS–PAGE and immuno and lectin blots of BacSBP expressed in Sf9 cells. (A) 2.5 μg BacSBP Jacalin blot. (B) 2.5 μg BacSBP WGA blot. (C) 2.5 μg BacSBP Con A blot. (D) Lane 1, 2.5 μg BacSBP immunoblot; lane 2, 2.5 μg plasma hSBP/hABP immunoblot. (E) Coomassie stained gel; lane 1, 2.5 μg BacSBP; lane 2, 10 μg plasma hSBP/hABP; lane 3, Bio-Rad molecular weight standards.

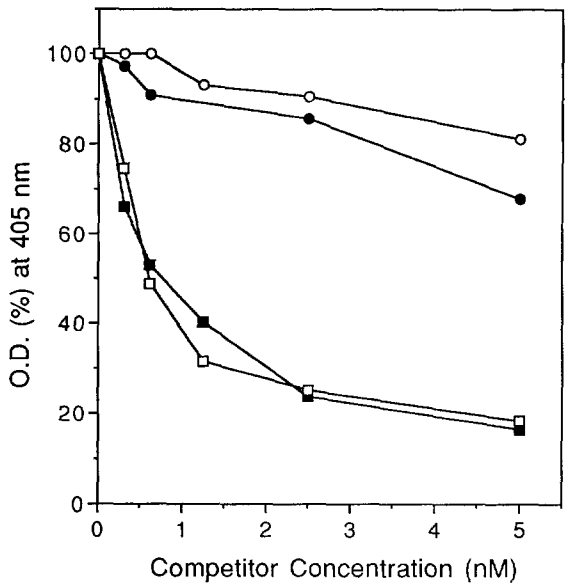

Fig. 4. Analysis of conformational epitopes by competitive strepavidin-biotin ELISA. The polyclonal antibody was incubated with 0.1 nM biotinylated SBP and mixed with increasing concentrations of competitor SBP: native plasma hSBP/hABP (■); BacSBP (□); plasma hSBP/hABP heated at 60°C for 1 h, (●); BacSBP heated at 60°C for 1 h, (○).

SBP/ABP compete equally well with biotinylated SBP for polyclonal antiSBP. As expected, when both proteins are denatured at 60°C for 1 h, competition is equally reduced.

The partial aminoterminal sequence of purified BacSBP was determined by automated Edman degradation. The following residues were obtained at each turn: NH_2-L(R)PVLP(t)Q-A(H)DPP. The putative serine at turn 9, indicated by the dash, was too low to be positively identified. Also, arginine and histidine were not determined. Threonine at turn 7 was present at very low level but still detectable which corroborates the immunoblots of Fig. 3(A–D) providing evidence for the presence of a small fraction of unglycosylated BacSBP molecules. The aminoterminal sequence of BacSBP is therefore identical to the published sequence of human plasma SBP except for the aminoterminal leucine which had been cleaved off in the original amino acid sequence determination [11].

DISCUSSION

The results presented here indicate that Sf9 and High Five cells, when infected with recombinant SBP baculovirus, express SBP at high levels either in plated cells or cells grown in suspension. High Five cells express BacSBP at significantly higher levels than Sf9 cells, but the protein appears to be degraded after 4 days of infection and thus must be isolated from the medium prior to that time. The physicochemical and steroid-binding properties of BacSBP are essentially identical to those of human plasma SBP except for the monomeric molecular weight which is lower by about 4 kDa. Interestingly, the lower molecular weight of BacSBP could be explained by the lack of one of the two oligosaccharide side-chains attached at either Asn^{351} or Asn^{367} since one N-linked oligosaccharide side-chain of plasma hSBP has an approximate mass of 5.2 kDa. Reduction in molecular mass could also be due to the presence of shorter oligosaccharide side-chains; however, heterogeneity would have been observed if this were the case. Of note is the presence of a third lower molecular weight band shown in Fig. 1(D). This monomeric subunit does not appear to contain any oligosaccharide side-chains since the various lectins tested do not bind, as shown in Fig. 3(A–C) (the right portion of the lower band in this latter blot is somewhat difficult to interpret because of the high background). Thus, absence of the third band in the lectin-probed membranes, which supports the absence of both N-linked and O-linked oligosaccharide side-chains, indicates that the third band represents unglycosylated recombinant SBP. This interpretation is strengthened by the presence of a small amount of threonine at turn 7 in the Edman degradation of BacSBP. In the case of the plasma protein, there is a "hole" at turn 7 supporting the presence of the O-linked chain at that residue [11]. Fully-deglycosylated SBP retains steroid-binding activity as indicated by the enzymatic removal of the oligosaccharide side-chains [25] and from recent data obtained on SBP expressed in bacteria (Sui and Petra, unpublished results). Thus, we believe that the oligosaccharide side-chains do not play a major role in the folding of the active conformation of SBP (vide infra). In contrast, we have shown that the sequence segment encompassing residues 130–143 not only forms part of the steroid-binding site [2, 19], which is likely stabilized by calcium [28], but also contributes to conformational stability by providing some binding energy to subunit dimerization [29].

Since the loss of specific activity resulting from replacing an amino acid by site-directed mutagenesis is often difficult to interpret in terms of a structure–function relationship, we have developed an ELISA for detecting a change in global conformation which, if it occurs, would likely lead to a loss of activity. The approach is similar to a radioimmunoassay recently-developed [30] but is more sensitive, easier to use, and less hazardous. The procedure, tested here for comparing plasma and recombinant SBPs, uses a nonradioactive streptavidin–biotin amplification protocol. Because both proteins compete equally-well with biotinylated SBP for polyclonal anti-SBP binding sites (Fig. 4), the results show that the conformational epitopes of each protein are probably similar. Since one would expect the epitopes recognized by the polyclonal antibody to be spread out over the entire surface of the dimeric molecule, the data of Fig. 4 strongly suggest that both proteins have similar three-dimensional structures and folding pathways. Although the interpretation could be problematic if most of the conformational epitopes were localized in a small region of the SBP molecule, we believe that that

possibility is unlikely because of the large size of dimeric SBP and the fact that the antibody used in the ELISA is polyclonal [31]. Nonetheless, the question is presently being addressed by repeating the procedure with a mixture of monoclonal antibodies recently isolated in our laboratory. Competition of heat-denatured BacSBP and hSBP/hABP with biotinylated SBP is also equally-reduced (Fig. 4) suggesting that the conformational epitopes are equally lost by thermodenaturation, further indicating that both proteins fold similarly. The remaining 30% competition, shown in Fig. 4, corresponds to amino acid sequence specific epitopes which would be unaffected by unfolding of the proteins. Preliminary results indicate that this procedure is valuable for detecting changes in conformation following site-directed mutagenesis (to be published) which should allow a more refined structural interpretation for explaining losses of activity.

In summary, recombinant BacSBP represents an excellent model for resolving questions on the structure and function of hSBP/hABP. This finding, along with more recent observations on the expression of SBP in bacteria (Sui and Petra, unpublished data), will permit the preparative isolation of glycosylated and deglycosylated recombinant SBP to allow characterization of SBP mutants and to resume efforts in crystallization. The crystals of plasma SBP obtained so-far were found unsuitable for X-ray diffraction. Since BacSBP is not microheterogeneous at the aminoterminus and contains less carbohydrate, we anticipate that crystallization trials of BacSBP will yield protein crystals amenable to X-ray diffraction. The expression system will facilitate characterization of the steroid-binding site and allow to establish the role of the alternating leucine sequence (ALS) motif [2, 11]. We have postulated that dimerization occurs through the interdigital interaction of the ALS residues to form a leucine "β-zipper". Such a structural motif has now been found in interleukin-1β [32]. Finally, the interaction of BacSBP and its mutants with the SBP membrane receptor will allow to pursue the role of SBP in cell steroid uptake.

Acknowledgements—The authors wish to thank Kenneth A. Walsh and Frederick S. Hagen for their advice and continuous interest in this project. We also thank Santosh Kumar for performing amino sequence analyses. This work was supported by NIH Grant HD-13956 (P.H.P.).

REFERENCES

1. Westphal U.: *Steroid–Protein Interaction II. Monograph on Endocrinology. Vol. 27.* Springer-Verlag, New York (1986).
2. Petra P. H. The plasma sex steroid-binding protein (SBP or SHBG). A critical review of recent developments on the structure, molecular biology, and function. *J. Steroid Biochem. Molec. Biol.* **40** (1991) 735–753.
3. Petra P. H., Stanczyk F. Z., Namkung P. C., Fritz M. A. and Novy M. J.: Direct influence of the sex steroid-binding protein of plasma on the metabolic clearance rate of testosterone. *J. Steroid Biochem.* **22** (1985) 739–746.
4. Plymate S. R., Namkung P. C., Matej L. A. and Petra P. H.: Direct effect of plasma sex hormone binding globulin on the metabolic clearance rate of 17β-estradiol in the primate. *J. Steroid Biochem.* **36** (1990) 311–317.
5. Hryb D. J., Khan M. S. and Rosner W.: Testosterone-estradiol binding globulin binds to human prostatic cell membranes. *Biochem. Biophys. Res. Commun.* **128** (1985) 432–440.
6. Fortunati N., Fissore F., Fazzari A., Berta L., Giudici M. and Frairia R.: Sex steroid binding protein interacts with a specific receptor on human pre-menopausal endometrium membrane: modulating effects of estradiol. *Steroids* **56** (1991) 341–346.
7. Porto C. S., Gunsalus G. L., Bardin C. W., Phillips D. M. and Musto N. A.: Receptor-mediated endocytosis of an extracellular steroid-binding protein (TeBG) in MCF-7 human breast cancer cells. *Endocrinology* **129** (1991) 436–445.
8. Fortunati N., Fissore F., Fazzari A., Berta L., Benedusi-Pagliano E. and Frairia R.: Biological relevance of the interaction between sex steroid binding protein and its specific receptor of MCF-7 cells: effects on the estradiol-induced cell proliferation. *J. Steroid Biochem. Molec. Biol.* **45** (1993) 435–444.
9. Bordin S. and Petra P. H.: Immunocytochemical localization of the plasma sex steroid-binding protein in tissues of the adult male monkey, *Macaca nemestrina*. *Proc. Natn. Acad. Sci. U.S.A.* 77 (1980) 5678–5682.
10. Gerard A., Egloff M., Gerard H., Harate E., Domingo M., Gueant J. L., Dang C. D. and Degrelle H.: Internalization of human sex steroid-binding protein in the monkey epididymis. *J. Molec. Endocrinol.* **5** (1990) 239–251.
11. Walsh K. A., Titani K., Kumar 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.
12. Gershagen S., Lundwall A. and Fernlund P. Characterization of the human sex hormone binding globulin gene and demonstration of two transcripts in both liver and testis. *Nucl. Acid Res.* **17** (1989) 9245–9258.
13. Hammond G. L., Underhill D. A., Rykse H. M. and Smith C. L.: The human sex hormone-binding globulin gene contains exons for androgen-binding protein and two other testicular messenger RNAs. *Molec. Endocrinol.* **3** (1989) 1869–1876.
14. Petra P. H., Titani K., Walsh K. A., Joseph D. R., Hall S. H. and French F. S.: Comparison of the amino acid sequence of the sex steroid-binding protein of human plasma with that of the androgen-binding protein of rat testis. In *Colloque/INSERM* (Edited by Forest M. G. and Pugeat M.). John Libbey, London/Paris. Vol. 149 (1986) pp. 137–142.
15. Joseph D. and Lawrence W.: Mutagenesis of essential functional residues of rat androgen-binding protein/sex hormone-binding globulin. *Molec. Endocrinol.* 7 (1993) 488–496.
16. Mickelson K. E. and Petra P. H.: A filter assay for the sex steroid binding protein of human serum. *FEBS Lett.* **44** (1975) 34–38.
17. Shanbhag V. P. and Sodergard R.: The temperature dependence of the binding of 5α-dihydrotestosterone, testosterone, and estradiol to the sex hormone binding globulin of human plasma. *J. Steroid Biochem.* **24** (1986) 549–555.
18 Hagen F. S., Arguelles C., Sui L. M., Zhang W., Seidel P. R., Conroy S. C. and Petra P. H.: Mammalian expression of the human sex steroid-binding protein of plasma (SBP or SHBG) and testis (ABP). Characterization of the recombinant protein. *FEBS Lett.* **299** (1992) 23–27.
19. Sui L. M., Cheung A. W. C., Namkung P. C. and Petra P. H.: Localization of the steroid-binding site of the human sex steroid-binding protein of plasma (SBP or SHBG) by site-directed mutagenesis. *FEBS Lett.* **310** (1992) 115–118.
20. Bocchinfuso W. P., Warmels-Rodenhiser S. and Hammond G. L.: Expression and differential glycosylation of human sex hormone-binding globulin by mammalian cell lines. *Molec. Endocrinol.* **5** (1991) 1723–1729.
21. Miller L. K.: Baculoviruses as gene expression vectors. *Ann. Rev. Microbiol.* **42** (1988) 177–199.
22. Petra P. H. and Lewis J.: Modification in the purification of the sex steroid-binding protein of human serum by affinity chromatography. *Anal. Biochem.* **105** (1980) 165–169.
23. Namkung P. C., Kumar S., Walsh K. A. and Petra P. H.: Identification of lysine-134 in the steroid binding site of the sex steroid binding protein of human plasma. *J. Biol. Chem.* **265** (1990) 18,345–18,350.
24. Petra P. H., Namkung P. C., Senear D. F., McCrae D. A., Rousslang K. W., Teller D. C. and Ross J. B. A.: Molecular characterization of the sex steroid-binding protein (SBP) of plasma. Re-examination of rabbit SBP and comparison with the human, macaque, and baboon proteins. *J. Steroid Biochem.* **25** (1986) 191–200.

25. Petra P. H., Griffin P. R., Yates III J. R., Moore K. and Zhang W.: The complete deglycosylation of native sex steroid-binding protein of human and rabbit plasma (SBP or SHBG). Effects on the steroid-binding activity. *Prot. Sci.* **1** (1992) 902–909.
26. Mickelson K. E. and Petra P. H.: Purification and physico-chemical characterization of the sex steroid-binding protein of rabbit serum. Comparison with the human protein. *J. Biol. Chem.* **253** (1978) 5293–5298.
27. Hunkapiller M. W., Hewick R. M., Dreyer W. J. and Hood L. E.: High-sensitivity sequencing with a gas-phase sequenator. *Meth. Enzymol.* **91** (1983) 399–413.
28. Ross J. B. A., Contino P. B. and Petra P. H.: Observation and quantitation of metal-binding sites in the sex steroid-binding protein of human and rabbit sera using the luminescent probe Terbium. *J. Prot. Chem.* **4** (1985) 299–304.
29. Casali E., Petra P. H. and Ross J. B. A.: Fluorescence investigation of the sex steroid-binding protein of rabbit serum: steroid-binding and subunit dissociation. *Biochemistry* **29** (1990) 9335–9343.
30. Bocchinfuso W. P., Warmels-Rodenhiser S. and Hammond G. L.: Structure/function analysis of human sex hormone-binding globulin by site-directed mutagenesis. *FEBS Lett.* **301** (1992) 227–230.
31. Bordin S., Lewis J. and Petra P. H.: Monospecific antibodies to the sex steroid-binding protein of human and rabbit serum. *Biochem. Biophys. Res. Commun.* **85** (1978) 391–401.
32. Gronenberg A. M. and Clore G. M.: Experimental support for the "Hydrophobic Zipper" hypothesis. *Science* **263** (1994) 536.