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Hormonal and immunological aspects of the phylogeny of sex steroid binding plasma protein

(estradiol/dihydrotestosterone/α-fetoprotein)

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ABSTRACT **Sex steroid binding plasma protein (Sbp) in man and in monkeys binds the androgens dihydrotestosterone and testosterone and the estrogen estradiol with high affinity (K_d ≈0.5, 1, and 2 nM, respectively). Detailed studies of steroid binding specificity give the same results in all primates, except that in humans and chimpanzees estrone does not compete for dihydrotestosterone binding. In other mammals, Sbps of Artiodactyla and Lagomorpha have the same range of affinities for androgens but they do not bind estradiol to any significant extent (K_d >280 nM). The dog has an unusual Sbp (K_d for dihydrotestosterone, 7.1 nM; for estradiol, 125 nM), and rodents do not have a specific dihydrotestosterone-binding plasma protein. Gel filtration and immunoelectrophoretic experiments have been performed with a monospecific antiserum against human Sbp. The results indicate variable crossreactivities with Sbps of primates (from complete in chimpanzee and gorilla to weak in Prosimii). No crossreaction was observed with specific androgen-binding plasma proteins of other species. These results suggest the evolutionary emergence of bifunctional Sbp.**

Sex steroid binding plasma protein (Sbp) was first described in the human (h-Sbp) (1). It was initially called "Protéine Liant la Testostérone" (Plt) (2) because of its high affinity binding of the typical androgens testosterone and 5α-dihydrotestosterone (Dht) with a K_d of ≈1 nM at 0–4°C, but was renamed when the binding of estradiol (3–7), presumably to the same site, was also observed. It is also known as sex hormone binding globulin (Shbg) (8) or testosterone-estradiol binding globulin (Tebg) (9). Sbp possibly functions as a reservoir/buffer, influencing free (active) hormone concentration in the plasma (10) but, as in the case for other high-affinity hormone binding plasma proteins such as thyroxine-binding globulin or corticosteroid-binding globulin, its physiological role is poorly understood. Progress has been made in the purification of h-Sbp (11–13), and the preparation of monospecific antibodies (14, 15) will presumably permit further understanding of its mode of action. Such antibodies have been used in this study, together with binding studies of Dht and estradiol, to investigate some phylogenic aspects of Sbps.

MATERIAL AND METHODS

Hormones. [^{3}H]Dht (specific activity, 55 Ci/mmol; 1 Ci = 3.7×10^{10} becquérels), [^{3}H]testosterone (specific activity, 48 Ci/mmol) and [^{3}H]estradiol (specific activity, 48 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, England. The radiochemical purity was checked monthly by thin-layer chromatography in benzene/acetone, 85:15 (vol/vol) for dihydrotestosterone and testosterone and benzene/ethyl acetate, 1:1 (vol/vol) for estradiol. Nonradioactive steroids were a gift of Roussel-Uclaf (Romainville) (guaranteed 99% pure).

Chemicals and Animals. Tubing [Visking–Nojax, 8/32 in. (6.4 mm); from Union Carbide Corporation, New York] was used in equilibrium dialyses. Agarose (Indubiose A 37) was purchased from l'Industrie Biologique Française (Paris), Ultrogel AcA 34 was from LKB (Uppsala, Sweden), and Freund's complete and incomplete adjuvants were from Difco. All other products were reagent grade.

Blood was obtained from rabbits (Fauves de Bourgogne), chickens (White Leghorn), rats (Wistar), and *Xenopus laevis* (South African Snake Farm) currently used in our laboratory. Primate samples were gifts from colleagues mentioned in the acknowledgements; we obtained sera from chimpanzees, gorillas, four baboon species (*Papio papio, P. anubis, P. cynocephalus,* and *P. hamadryas*), two species of Macaca [*Macaca mulatta* (rhesus) and *M. fascicularis* (*cynomulgus*)], two species of new world monkeys [Cebidae: *Ateles paniscus* (spider monkey) and *Lagothrix lagothricha* (woolly monkey)], one other Cebidae [*Saimiri sciureus* (squirrel monkey)], one species of Callithricidae [*Callithrix jacchus* (Marmoset)], and one Prosimii (*Microcebus murinus*). All other blood samples were obtained from the Institut National de la Recherche Agronomique (Nouzilly, France).

Pure Sbp. Pure h-Sbp was obtained as described (13) from plasma of women in late pregnancy. Dht-binding activity of the pure protein showed a greater than 4200-fold increase over total plasma binding, corresponding to ≈15 mg of h-Sbp per liter; this was estimated on the basis of one binding site per 100,000 daltons—an approximation near to the probable-but-still-controversial value of the molecular weight of the native protein (as explained in ref. 16). Homogeneity was assessed by polyacrylamide gel electrophoresis in nondenaturing and sodium dodecyl sulfate buffer systems at various acrylamide concentrations and by immunoelectrophoresis analysis against a crude anti-h-Sbp antiserum (14). The h-Sbp was stored frozen at 350–500 μg of protein per ml in 0.05 M Tris/0.05 M $CaCl_2$/0.2 M NaCl/HCl buffer, pH 7.4, containing 100 nM Dht and 10% vol/vol glycerol. Pure h-Sbp concentration was determined by fluorimetry with excitation and emission wavelengths of 290 and 330 nm, respectively; transferrin (200 μg/ml) was used as standard.

Immunization. Adult male rabbits were injected (under the nail of the hind toepads) with 50–100 μg of h-Sbp emulsified in 0.5 ml of Freund's complete adjuvant. A booster injection of the same amount of Sbp emulsified in Freund's incomplete

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Abbreviations: (h-)Sbp(s), (human) sex steroid binding plasma protein(s); Dht, 5α-dihydrotestosterone; r-Sbp, rabbit Sbp.

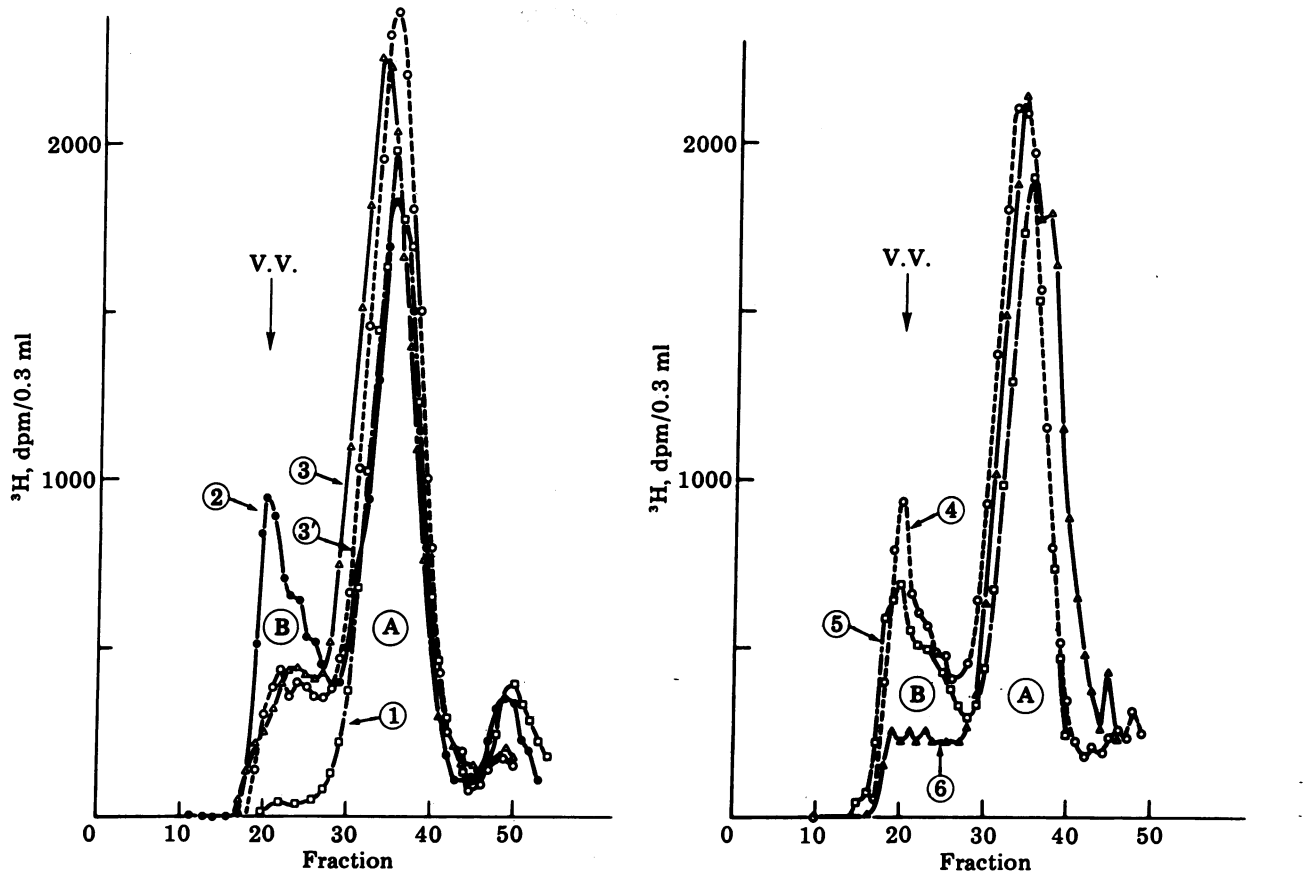

FIG. 1. Ultrogel AcA 34 filtration of [^{3}H]Dht–Sbp–anti-h-Sbp antibody complexes. Ultrogel AcA 34 columns (20 × 0.5 cm) were equilibrated with 0.05 M Tris/0.2 M NaCl/0.05 M $CaCl_2$/HCl (pH 7.4) and 0.3-ml fractions were collected. The following incubates were filtered. (*Left*) Curve 1:50 μl of partially purified human late pregnancy plasma incubated with 10 nM [^{3}H]Dht at 4°C for 3 hr (□--□); curve 2: as for curve 1 plus 100 μl of specific anti-h-Sbp antiserum incubated at 4°C for 24 hr (●—●); curve 3: as for curve 2 plus 50 μl of unlabeled human late pregnancy plasma (△—△); curve 3: as for curve 2 plus 50 μl of *P. papio* plasma. Same results were obtained with rhesus monkey, woolly monkey, and chimpanzee plasmas (○---○); (*Right*) curve 4: as for curve 2 plus 50 μl of dog plasma (○---○); curve 5: as for curve 2 plus 50 μl of rat plasma (□--□); curve 6: as for curve 2 but the monospecific rabbit anti-h-Sbp antiserum was replaced by 100 μl of preimmune rabbit serum (△—△). V.V., void volume.

adjuvant was administered 5 weeks later in a similar manner. Blood was collected every 2 weeks for 6 months by section of the marginal ear vein, and the collected sera were frozen in 500-μl aliquots at −20°C.

Anti-h-Sbp antibodies obtained after injection of pure h-Sbp were found to be monospecific without further purification (16).

Detection of Antibodies to h-Sbp by Gel Filtration. Gel filration on Ultrogel AcA 34 columns (20 × 0.5 cm) was used to detect the presence of anti-h-Sbp antibodies (14). Partially purified h-Sbp was prepared by precipitation from human late-pregnancy plasma at 42% ammonium sulfate saturation and incubated with 10 nM [^{3}H]Dht for 3 hr at 4°C. The complexes were then exposed to the anti-h-Sbp antiserum for 24 hr at 4°C. As shown in Fig. 1, complexes of [^{3}H]Dht–h-Sbp were eluted in the void volume of the column in the presence of antiserum (curve 2, peak B), whereas no labeled material was eluted in the void volume in the absence of antiserum (curve 1, peak A). When anti-h-Sbp antiserum was replaced by preimmune rabbit serum, only a small amount of radioactivity was eluted in the void volume (curve 6, peak B). No further decrease was observed when an excess of unlabeled Dht was added to the incubate (not shown). In fact, the same low level of radioactivity was observed in the void volume, when excess nonradioactive Dht was added to the [^{3}H]Dht incubation in the presence of antiserum (not shown). This residual level of radioactivity may be due to the trapping of some nonspecific binding entity by a nonspecific component of the rabbit serum. Rabbit (nonimmune) serum contains a Dht-binding Sbp (r-Sbp) that displays an affinity similar to that of h-Sbp (17–20), and this protein is automatically included in the tested preparation; this r-Sbp is eluted in peak A of the column, binds some radioactive hormone, and contributes eventually to the decrease of peak B. The peak B observed after incubation of the rabbit antiserum with radioactive Dht–h-Sbp (curve 2) was decreased markedly when 50 μl of human plasma were added to the mixture together with the antiserum (compare curves 2 and 3).

Immunological Crossreactivity Studied by Electrophoresis. In order to demonstrate the immunological crossreactivity of Sbps, samples of plasma or serum were subjected to electrophoresis in 1.3% agar gel (14). Pure h-Sbp was introduced into a second well (Fig. 2). The anti-h-Sbp antiserum was then deposited in the trough and immunodiffusion allowed to take place at room temperature for 48 hr. Total immunological identity was observed with the corresponding protein of human late pregnancy plasma (Fig. 2 *Upper*), which demonstrated that the purification had not altered the immunological determinants of the molecule.

Hormone-Binding Experiments. Equilibrium dialysis experiments were performed. The plasma or serum samples were stripped of endogenous steroid(s) by using charcoal (5 mg/ml for 10 min at 37°C), and diluted with 0.05 M Tris/0.05 M $CaCl_2$/0.2 M NaCl/HCl, pH 7.4; 1-ml bags were dialyzed

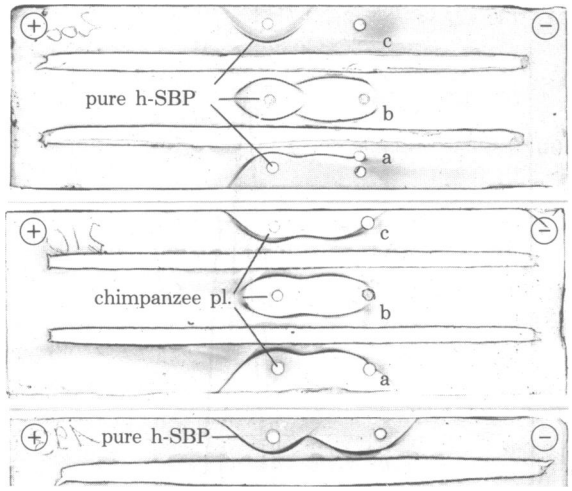

FIG. 2. Immunoelectrophoresis studies of Sbps. Antigen samples (right wells) were first submitted to electrophoresis as described (14). Then the troughs and the left wells were cut off and filled with the monospecific anti-h-Sbp antiserum (*Top*), pure h-Sbp (600 μg/ml) (*Bottom*) and chimpanzee plasma (*Middle*). Precipitation arcs were allowed to develop during 48 hr at 25°C. (*Top*) Human late pregnancy plasma (a), marmoset plasma (b), and ewe plasma (c) show total, partial, and no immunological crossreactivity with pure h-Sbp, respectively. (*Middle*) *P. papio* plasma (a), pure h-Sbp (b), and human late pregnancy plasma (c) show total immunological crossreactivity with chimpanzee Sbp. (*Bottom*) A weak and partial immunological identity occurs between *P. papio* Sbp and pure h-Sbp.

against 15 ml of Tris buffer containing 1 nM of [^{3}H]-labeled hormone, either alone or together with 100 nM of unlabeled steroid, in order to detect specific binding (calculated by subtracting the nonspecific value obtained in the presence of nonradioactive ligand). After 24 hr at 4°C with constant stirring, 0.2-ml aliquots of both internal and external solutions were measured in duplicate for radioactivity. The K_d and concentration of binding sites were determined by using increasing amounts of [^{3}H]-labeled hormone (0.1–50 nM), and the results were plotted according to the method of Scatchard (21) as modified by Rosenthal (22). The mean (±95% confidence limits) of K_d and of the number of sites are reported.

RESULTS

Immunological Studies. When monkey plasma was substituted for human plasma in gel filtration experiments (Fig. 1, curve 3′) the magnitude of peak B decreased, but such a decrease was not observed in similar experiments when dog plasma (containing Sbp of weaker affinity for Dht) or rat plasma (not containing Sbp) was used instead of primate plasma (curves 4 and 5). Moreover, when plasmas of monkeys (*P. papio*, chimpanzee, and rhesus monkey) previously labeled with [^{3}H]Dht were incubated with the antiserum, a peak B was observed similar to that of curve 2, whereas this was not the case with dog or chicken plasma. Together these findings suggested that antibodies against h-Sbp are able to interact with both human and monkey Dht-binding Sbps.

When monkey plasmas were studied by immunoelectrophoresis, precipitation arcs were obtained, as opposed to that observed with plasmas of other animals (Fig. 2 *Upper;* Table 1). However, only the chimpanzee and the gorilla (data not shown) Sbps gave total identity crossreaction with anti-h-Sbp antiserum (Fig. 2 *Middle*). In addition, although immunological crossreactivity with baboons (Fig. 2 *Bottom*) was partial, it was strong; with other monkeys, the partial immunological identity essentially followed the primate phylogeny (Fig. 2; Tables 1 and 2). Finally, the number of common antigenic determinants with h-Sbp was the smallest with Prosimii.

Table 1. Binding of androgens and estradiol to Sbps

	Immunoreactivity with anti-h Sbp antibodies	K_d, nM			K_d ratio, E_2/Dht	Binding site concentration in plasma, nM
		Dht	T	E_2		
Human	To	0.4	0.8	2.0	5.0	M 38 F 70
Chimpanzee	To	0.5	0.8	2.2	4.4	M 30 F 60
Gorilla	To	0.4	0.9	1.7	4.2	*
Baboon	Pa	0.5	1.0	2.5	5.0	F 200
Rhesus monkey	Pa	0.8	2.4	4.2	5.3	F 155
Bull	–	2.5	4.8	333	133	M 200
Goat	–	0.9	2.3	280	311	M 100
Pig	–	–	–	–		–
Ram	–	1.8	3.5	534	295	M 125
Dog	–	7.1	25	125	18	M 185
Horse	–	–	–	ND		–
Rabbit	–	0.5	3.0	295	590	M 500
Rat	–	–	–	–†		–
Mouse	–	–	–	–†		–
Guinea pig	–	–	–	ND		–
Rat α-FP	–	–	–	16		
Human α-FP	–	–	–	–		

K_ds of testosterone (T), dihydrotestosterone (Dht), and estradiol (E_2), were measured on the serum or plasma at 4°C. Concentrations were determined from binding studies with [^{3}H]-labeled compounds in plasma samples from males (M) or females (F). –, Undetectable; ND, not done; α-FP, α-fetoprotein; To and Pa, total and partial crossreactivity.

* Only young gorillas were available: a 7-year-old female and a 3-year-old male; concentrations of what were 250 and 120 nM, respectively.

†, Adult.

Table 2. Immunological crossreactivity of primate Sbps with anti-h-Sbp antibody

	Anthropoidae								Prosimii
	Old World monkeys						New World monkeys		
	Hominidae	Pongidae	Cercopithecidae		Cebidae	Callithrichidae	Cebidae		Cherogaleidae
	Man	Chimpanzee	Baboon	Macaca	Squirrel monkey	Marmoset	Woolly monkey	Spider monkey	Microcebus
Man		To	Pa★★★★	Pa★★★	Pa★★	Pa★★	Pa★★	Pa★★	Pa★
Chimpanzee	To		To	To	Pa★★	Pa★★	Pa★★★	—	—
Baboon	Pa★★★	To		To	Pa★★★	Pa★★★	Pa★★★	Pa★★★	—
Macaca	Pa★★★	To	To		To	Pa★★★★	Pa★★★★	Pa★★★★	Pa★
Squirrel monkey	Pa★★	Pa★★	Pa★★★	To		To	To	To	—
Marmoset	Pa★★	Pa★★	Pa★★★	Pa★★★★	To		To	Pa★★★★	—
Woolly monkey	Pa★★★	Pa★★★	Pa★★★	Pa★★★★	To	To		To	Pa★
Spider monkey	Pa★★★	—	Pa★★★	Pa★★★★	To	Pa★★★★	To		—
Microcebus	Pa★	—	—	Pa★	—	—	Pa★	—	

To, total identity (see also Fig. 2); Pa, partial identity; ★★★★ to ★, strong to weak or partial crossreactivity.

Studies of Hormone-Binding Specificity. High-affinity specific Dht binding was observed in monkeys, in some Artiodactyla (not in the pig), and in the rabbit, but not in the horse or in rodents (Table 1). The K_d of Sbps was slightly lower in primates than in Artiodactyla. Interestingly, binding studies with estradiol also indicated a difference between primates and other species. Binding affinity for estradiol was high in all primates tested, with a ratio of the K_ds of estradiol and Dht in the order of 5. The specific binding of estradiol by plasma of other species was weak, with a K_d in the range of 0.1 μM or higher. The dog is unusual among nonprimate mammals: its Sbp binds testosterone and Dht with an affinity for androgens lower than that in primates and binds estradiol with an affinity higher than in other nonprimate species.

In view of the preceding results, we investigated in detail the binding specificity of monkey Sbps. The binding test consisted of adding the competitive hormone at 10 nM to 0.1 nM [^{3}H]Dht and dialyzing at ≈4°C for 18–20 hr. The low concentration of steroid minimized uncontrolled metabolic transformations, which may occur even at low temperatures with high substrate concentrations. All primate Sbps showed no specific binding of progesterone, cortisol, estriol (a natural steroidal estrogen metabolite), or diethylstilbestrol (a synthetic nonsteroidal estrogen) (Table 3). Estradiol binding was identical in all species—weaker than that for testosterone. Curiously, estrone was a good competitor of [^{3}H]Dht binding in tested monkeys [including gorilla (data not shown)] but not in man or chimpanzee. In addition to binding Dht, primate Sbps bound testosterone and 3α/β-5α-androstanediols with high affinity. Finally, the plasma concentrations of Sbp binding sites were similar in man and chimpanzee, being less than 0.1 μM, as opposed to those in other monkeys, in which the concentrations were 2–5 times greater (Table 1).

DISCUSSION

These binding experiments are essentially in agreement with those of other authors (23–28), including the Dht binding observed in several *Macacas* (29, 30). Whereas a specific Dht-binding protein has been demonstrated in primates and several lower species, these immunological studies indicate reactivity only between monkey sera or plasmas and anti-h-Sbp antibodies.

Interestingly, binding studies with estradiol also indicate a difference between primates and other species and, in spite of the lower affinity for the androgens, the ratio of K_ds for estradiol and Dht was higher for other species than for primates. The case of the dog is unusual; the ratio of the K_ds for estradiol and dihydrotestosterone in the dog is not very far from that observed in primates (Table 1). This may be related to the spontaneous occurrence of a particular form of benign prostatic hypertrophy in the dog, which is used as an animal model for the human disease. The rat does not have a specific testosterone-binding

Table 3. Binding of steroid hormones by Sbps in primates

Steroid	Man	Chimpanzee	Baboon	Rhesus monkey	Squirrel monkey	Marmoset
Dht	100	100	100	100	100	100
T	79	92	83	87	81	77
E_2	33	57	38	55	60	79
3α-diol	101	104	98	97	96	97
3β-diol	104	101	96	100	91	99
Des	0	0	0	0	0	0
E_1	0	0	30	20	42	52
E_3	0	0	4	0	0	16
Cortisol	0	3	0	0	0	0
Progesterone	0	0	0	0	0	3
K_d, nM at 4°C	(M) 0.43 ± 0.06 (F-G) 0.43 ± 0.06	(F) 0.48 ± 0.05	(F) 0.50 ± 0.06	(F) 0.8 ± 0.1	(F) 1.1 ± 0.1	(F) 1.1 ± 0.1
Plasma concentration, nM	(F) 70 ± 30 (F-G) 700 ± 185	(F) 60 ± 15	(F) 200 ± 67	(F) 155 ± 75	(F) 259 ± 133	(F) 108 ± 81

T, testosterone; 3α-diol, 5α-androstane-3α,17β-diol; 3β-diol, 5α-androstane-3β,17β-diol; E_1, estrone; E_2, estradiol; E_3, estriol (1,3,5-estratriene-3,16α,17β-triol); Des, diethylstilbestrol; M, males; F, females; F-G, pregnant females. The competitive binding test was performed with 0.1 nM [^{3}H]Dht and 10 nM of nonradioactive competitor. The decrease of bound radioactivity measured after addition of competitor was relative to that measured after Dht addition ("100"). K_d and concentration values are mean ± SEM.

plasma protein, but the fetal blood contains an estrogen-binding α-fetoprotein (31). The human α-fetoprotein does not crossreact with the antibodies against h-Sbp [and incidentally does not bind estradiol (unpublished data)] and, conversely, antibodies against either human (unpublished data) or rat (32) α-fetoprotein do not crossreact with h-Sbp.

Detailed studies with monkey sera indicate that the human and the chimpanzee Sbps appear to be very similar and their concentrations are identical. The differences in immunoreactivity and estrogen binding between the Sbps of primates and other species suggest that a specific role for plasma proteins emerged with evolution and is possibly related to a fine regulatory function of androgen/estrogen equilibrium in the blood (33). The relatively weak affinity ($K_d \approx 0.1$ μM) of plasma protein in fish for the triple binding of testosterone, estradiol, and progesterone (34, 35) may be assigned to another category of proteins. It is interesting to note that the situation may be different for the high-affinity estrogen-binding receptors. Antibodies against the calf (uterus) receptor crossreact with the chick (oviduct) receptor, which suggests more evolutionary conservation for this intracellular protein than for the plasma protein (36).

In conclusion, the very systematic differences between testosterone and estradiol binding by Sbps have been correlated with their immunocrossreactivity using anti-h-Sbp antibodies. Testosterone binding may be considered as the basic feature of this circulating steroid(s) binding protein. It is remarkable that high-affinity estradiol binding is found only in primates, which alone have Sbps reacting with the anti-h-Sbp antibody. However, the physiological significance of such a precise mechanism for binding sex steroids escapes definitive understanding at the present time.

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