Arginine-140 and isoleucine-141 determine the 17 β -estradiol-binding specificity of the sex-steroid-binding protein (SBP, or SHBG) of human plasma

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This paper is dedicated to Prof. Hans Neurath on the occasion of his ninety-second birthday.

Abstract

Arginine-140 and isoleucine-141 were identified as key determinants of 17 β -estradiol (E₂) binding affinity of the sex-steroid-binding protein (SBP, or SHBG) of human plasma. Amino acid residues that differ between human and rabbit SBP sequences were replaced in the human protein and the products tested for lowered E₂ binding activity as are seen in the rabbit protein. Only mutants containing either R140K or I141L replacements display an E₂ equilibrium dissociation constant (K_d) higher than the wild type, reaching a value of 30 nM when both were present. The 5 α -dihydrotestosterone (DHT) equilibrium dissociation constant of these mutants was unaffected. The quadruple mutant M107I/I138V/R140K/I141L yielded an E₂ K_d of 65 nM, significantly closer to the 80 nM rabbit SBP E₂ K_d value. Although mutants containing the M107I and I138V replacements in the absence of R140K and I141L had normal E₂ K_ds, the presence of the M107I replacement in the quadruple mutant was necessary to obtain an accurate E₂ K_d value by competitive Scatchard analysis. Molecular modeling using coordinates for the recently determined N-terminal domain of human SBP revealed a significant shift of the F56 phenyl ring away from ring A of E₂ in mutant models containing the R140K and I141L replacements. We conclude that R140 and I141 are required for sustaining the right proximity of the phenyl ring of F56 to ring A of 17 β -estradiol, thus optimizing the E₂-binding affinity of human SBP.

Keywords: Sex-steroid-binding protein, SBP; sex-hormone-binding globulin, SHBG; dihydrotestosterone; testosterone; 17 β -estradiol; steroid-binding site

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Abbreviations: SBP, plasma sex steroid-binding protein; SHBG, sex hormone binding globulin; ABP, androgen binding protein; hSBP, human SBP; rSBP, rabbit SBP; DHT, 5 α -dihydrotestosterone; T, testosterone; E₂, 17 β -estradiol; Y57, tyrosine-57; M107, methionine-107; M139, methionine-139; I101, isoleucine-101; K134, lysine-134; Y57F, replacement of Y57 by phenylalanine at position 57; Y57A, replacement of Y57 by alanine at position 57; Y57T, replacement of Y57 by threonine at position 57; Y57G, replacement of Y57 by glycine at position 57; Y57L, replacement of Y57 by leucine at position 57; Y57S, replacement of Y57 by serine at position 57; M107I, replacement of M107 by isoleucine at position 107; M107L, replacement of M107 by leucine at position 107; M107T, replacement of M107 by threonine at position 107; M107A, replacement of M107 by alanine at position 107.

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Human sex-steroid-binding protein (SBP, also referred to as SHBG, sex-hormone-binding globulin) is a 93.4 kD homodimeric plasma glycoprotein, present in most species tested, that specifically binds one mole of testosterone (T), 5 α -dihydrotestosterone (DHT), or 17 β -estradiol (E₂) per mole dimer (Petra 1979, 1991; Westphal 1986; Joseph 1994). All three steroid hormones compete for the same binding site. The human SBP monomer contains 373 amino acid residues and three oligosaccharide side chains, one O-linked to T7 and two N-linked to N351 and N367 (Walsh et al. 1986). Removal of these side chains either enzymatically or genetically does not affect steroid-binding activity (Petra et al. 1992; Sui et al. 1999). Although SBP binds 2 moles of calcium per monomer (Ross et al. 1985), the metal does not appear to play a role in steroid binding or dimer-

ization (Sui et al. 1996); however, it could function in stabilizing the folding of certain regions of the protein, as indicated by the protection by calcium against heat denaturation (Rosner et al. 1974). Electron microscopy reveals that SBP has a rodlike structure, and calculations from circular dichroism measurements indicate that it contains 15% helix, 43% β -sheet, and 10–16% β -turns (Beck et al., 1997).

The physiological role of SBP is to regulate the bioavailability of sex steroid hormones to target tissues by controlling their metabolic clearance rates in plasma (Bardin and Lipsett 1967; Vermeulen et al. 1969; Petra et al. 1985; Plymate et al. 1990). The protein has also been proposed to play two additional roles, one in assisting the uptake of hormone by cells (Strel'chyonok et al. 1984; Pardridge 1988) and the other in signaling (Nakhla et al. 1990; Fissore et al. 1994).

Four amino acid side chains have been identified as functional in steroid binding, Y57 (Petra et al. 2000), M107 (Petra et al. 2000), M139 (Grenot et al. 1992; Sui et al. 1992), and K134 (Namkung et al. 1990); the first two residues only play a role in DHT binding (Petra et al. 2000). A recently published crystal structure of the N-terminal domain of human SBP (residues 13–188; Grishkovskaya et al. 2000) confirms the presence of M107 and M139 in the steroid-binding site; however, Y57 does not make direct contact with the ligand and the peptide region containing K134 did not exhibit electron density and could not be modeled. Because the calcium atoms are located ~ 20 Å from the steroid-binding site and ~ 18 Å from the subunit interface, they do not contribute directly to ligand binding and dimerization, as previously found (Sui et al. 1996). A most interesting finding is the presence of two steroid-binding sites located one in each truncated subunit resulting in a ligand-binding stoichiometry of 2 moles per dimer; this result is in contrast to that found for the native protein, which binds 1 mole ligand per mole dimer (Petra et al. 1986 and references cited therein).

The amino acid sequences of human and rabbit SBP⁴ are 80% identical (Walsh et al. 1986; Griffin et al. 1989; Lee et al. 1997). The steroid-binding site of the latter contains M133 (Kassab et al. 1998), as well as I101 (Petra et al. 2000), the homologs of M139 and M107 in human SBP (Walsh et al. 1986). The two proteins bind DHT with similar affinities but rabbit SBP has a markedly reduced affinity for E₂ (Rosner and Darmstadt 1973) with a K_d equal to 80 nM, compared to 4 nM for human SBP (Mickelson and

Petra 1978). Because both E₂ and DHT bind at the same site and the only major difference between the two steroids lies in the saturation and orientation of ring A, these data suggest that the structural motif recognizing ring A of E₂ in human SBP is significantly altered in rabbit SBP. We reasoned that this motif must be specified by residues that are among the 77 residues that are different in the two sequences (excluding the six residues at the N terminus of human SBP that are missing in the rabbit sequence). The experiments described here led to the identification of R140 and I141 as key determinants of E₂-binding specificity in human SBP.

Results

Site-directed mutagenesis

Rabbit SBP exhibits a 20-fold lower binding affinity for E₂ when compared to human SBP. To understand the determinants of this difference in affinity, rabbit SBP residues were inserted into the human construct where residues differed between the two sequences, and the products tested for loss of E₂-binding activity. There are 77 such residues (Fig. 1). Because the differences in structure between DHT and E₂ are small, it was expected that replacement of not more than two residues would likely be sufficient for obtaining a loss in E₂-binding activity. The search started by aligning the two sequences and dividing the set into segments containing from one to five different residues (Fig. 1; Table 1). A total of 56 replacements were made proceeding from the N terminus using combinatorial oligonucleotides coding for the changes in each selected segment. Replacements beyond L271 were not made because the C-terminal region of SBP is not thought to be involved in steroid-binding activity.

As shown in columns 3 and 4 of Table 1, except for the M107I mutants, all products had wild-type DHT K_d values, and most of them had wild-type E₂ K_d values, except for the ones containing the R140K and I141L replacements. Those with either replacement had an approximate 4-fold increase in the E₂ K_d (Fig. 2A,B) compared to wild type (Fig. 3B), while those with both R140K and I141L replacements showed a 10-fold increase (Fig. 3A); however, they still exhibited E₂ K_ds of about one-third the value of native rabbit SBP (Fig. 4B, Table 1). The presence of the I138V or L143V replacements in the mutants containing R140K and I141L had no influence on the DHT and E₂ K_ds. Taken at face value these findings suggested that other interactions could provide additional binding energy for ring A of E₂, thus necessitating making more mutations towards the C-terminal end of the protein or constructing new clones containing accumulated mutations for further increasing the E₂ K_d to match that of rabbit SBP. However, because the correct estimation of the E₂ K_d by competitive Scatchard analysis depends on the value of the DHT K_d determined in

⁴Three amino acid residues identified in a rabbit SBP cDNA clone were reported to be different (Lee et al. 1997) from those in the published sequence determined in our laboratory (Griffin et al. 1989). The cDNA deduced assignments were P295, S296, and Q307, as compared to S295, P296, and K307 in our sequence. The sequence of the segment containing these residues, originally determined by mass spectrometry (Griffin et al. 1989), was redetermined by Edman degradation. We confirm the cDNA assignments.

Fig. 1. Alignment of the amino acid sequences of rabbit and human SBP. The solid line denotes identical residues; the solid circle indicates the O-glycosylation site in human SBP; the asterisks indicate N-glycosylation sites.

the same experiment (see Materials and Methods), the apparently lower 30 nM value could be explained by the presence of methionine at position 107. Although replacement of M107 with isoleucine has no influence on the binding of E_2 in human SBP (Petra et al. 2000), methionine at position 107 in the human protein is required for maintaining the human SBP DHT K_d low at 0.42 nM (see Fig. 3B and the human M107I mutant in Table 1). Rabbit SBP, which contains isoleucine at the homologous position (Fig. 1), has a higher DHT K_d value (1.77 nM; Fig. 4B; Table 1) and that value is equal to that obtained for the human M107I (1.75 nM; Table 1). We therefore incorporated the M107I mutation into a new construct, isolated the M107I/I138V/R140K/I141L mutant, expressed it in COS-7 cells, and calculated the E_2 K_d by competitive Scatchard analysis. An E_2 K_d of 65 nM, a value closer to that of rabbit SBP (85 nM), and a DHT K_d of 1.75 nM equal to rabbit SBP were obtained (Fig. 4B). Because the I138V replacement had no effect on E_2 -binding affinity when present in any of the tested R140K and I141L mutants (Table 1), we conclude

that R140 and I141 are key amino acid residues in human SBP that determine the binding affinity of E_2 .

Molecular modeling

Molecular modeling was used in an attempt to find a structural explanation for the loss of E_2 binding in the mutants described above. The structures of E_2 and DHT and key residues surrounding each of them in the wild-type human SBP (Fig. 5) show that the primary reason for the higher K_d of estradiol versus DHT is the loss of the hydrogen bond between S42 O $^{\gamma 1}$ and O3 of DHT, as previously suggested (Grishkovskaya et al. 2000). The shape of the two steroids is nearly identical, except at the A ring. In E_2 the ring is aromatic and the oxygen moiety is a hydroxyl; whereas in DHT, ring A has a chair conformation and the oxygen moiety is a carbonyl. SBP appears to be optimally designed to bind this steroid, in that Ser 42 OG is positioned by an amide nitrogen-hydrogen bond that orients the serine OG so that its hydrogen can bond to O3 of ring A. Both the co-

Table 1. Equilibrium dissociation constants of human SBP mutants

Segment	Mutations Human to rabbit residues	K _d (nM) DHT	K _d (nM) E ₂
Human SBP	No mutation	0.42	4.22 ¹ , 4.79 ²
COS/Wt	No mutation	0.44 ⁸	3.83 ^{1,8} , 2.60 ²
Rabbit SBP	No mutation	1.77 ⁹	83.31 ^{3,9}
1–79	S9R/H11Q/P13S	0.79	3.71 ¹
	S19I/P22L	0.40	3.42 ¹
	K36R/I37L	0.44	4.79 ¹
	K36R/T38V/T40A	0.60	3.64 ¹
	N61S	0.43	2.97 ¹
80–108	L80M/H83P	0.66	3.37 ²
	R99S/E104H/E108R	0.39	4.62 ⁴
	E104H/M107I/E108R	2.33	3.57 ²
	M107I	1.75	3.55 ¹ , 2.92 ²
109–152	E119K	0.65	2.41 ²
	R125S/T132H/S133D/R135P	0.55	5.08 ²
	P130T/T132H/S133D/H136Q	0.35	4.48 ²
	I138V/R140K/I141L/L143V	0.52	23.37 ³
	I138V/R140K	0.56	17.00 ² , 16.11 ⁴
	I138V/I141L/L143V	0.59	12.07 ²
	I138V/R140K/I141L	0.70	21.2 ⁴
	R140K ⁵	0.46	18.45 ⁴
	I141L ⁶	0.67	16.31 ³
	L143V	0.59	3.62 ² , 5.17 ¹
	R140K/I141L/L143V ⁷	0.52	39.16 ⁴ , 31.79 ⁴
153–198	A150P/N152S	0.25	5.58 ¹
	D168G/K173P/E176Q	0.84	2.98 ²
	P182H/T183A/L185R	0.64	3.57 ²
	S192L/N193Q/L198F	0.51	4.41 ¹
199–243	N207S/R209Q	0.50	4.81 ²
	H215Q/A216T	0.66	3.12 ²
	G226E/Q229P/A230S/ A231E/H235R	0.66	5.45 ¹

¹ Estimated by competitive Scatchard analysis, using [1,2-³H]DHT and 8.00 nM radioinert E₂.
² Estimated by direct Scatchard analysis, using [6,7-³H]E₂. Average of 2–3 determinations.
³ Estimated by competitive Scatchard analysis, using [1,2-³H]DHT and 50.67 nM radioinert E₂.
⁴ Estimated by competitive Scatchard analysis, using [1,2-³H]DHT and 25.33 nM radioinert E₂.
⁵ See Fig. 2A.
⁶ See Fig. 2B.
⁷ See Fig. 3A.
⁸ See Fig. 3B.
⁹ See Fig. 4B.

planarity of the hydroxyl and likely presence of a hydrogen preclude optimal binding of E₂, even though most of the remaining van der Waal's interactions over the rest of the steroid are the same. Thus, in the absence of the Ser 42 OG-O3 hydrogen bond, other factors may become relatively more important, particularly the van der Waals contacts between the planar A-ring of E₂ and that of F56 (Fig. 5).

The structural changes in the modeled steroid-binding sites for the mutants are shown in Figure 6. The triple mutant M107I/R140K/I141L was modeled instead of the quadruple mutant M107I/I138V/R140K/I141L because the

I138V replacement had no effect on E₂ binding, whether it was present in mutants containing R140 or I141L replacements (Table 1). Figure 6A shows that, as expected, there are no structural differences between wild-type SBP and the triple mutant when DHT is bound in the site and that the dominant interaction is the H-bond between S42 OG and O3 of ring A of the steroid. The absence of a structural difference is consistent with the fact that a loss in DHT-binding affinity did not occur when these replacements were made.

Fig. 2. Determination of DHT and E₂ equilibrium dissociation constants of COS-expressed R140K and I141L human SBP mutants by direct and competitive Scatchard analysis at pH 7.4 and 4°C. (A) R140K, (solid circles) SBP samples (2 nM) incubated with a range of 1–10 nM [1,2-³H]DHT; (open circles) SBP samples (2 nM) incubated with 25.33 nM radioinert E₂ and a range of 1–10 nM [1,2-³H]DHT. (B) I141L, (solid circles) SBP samples (2 nM) incubated with a range of 1–10 nM [1,2-³H]DHT; (open circles) SBP samples (2 nM) incubated with 50.67 nM radioinert E₂ and a range of 1–10 nM [1,2-³H]DHT. The E₂ equilibrium dissociation constants (K_ds) were calculated as described in Materials and Methods.

Fig. 3. Determination of DHT and E₂ equilibrium dissociation constants (K_ds) of the COS-expressed R140K/I141L/L143V mutant and wild-type human SBP by direct and competitive Scatchard analysis at pH 7.4 and 4°C. (A) R140K/I141L/L143V, (solid circles) SBP samples (2 nM) incubated with a range of 1–10 nM [1,2-³H]DHT; (open circles) SBP samples (2 nM) incubated with 25.33 nM radioinert E₂ and a range of 1–10 nM [1,2-³H]DHT. (B) Human SBP, (solid circles) SBP samples (2 nM) incubated with a range of 1–10 nM [1,2-³H]DHT; (open circles) SBP samples (2 nM) incubated with 8.00 nM radioinert E₂ and a range of 1–10 nM [1,2-³H]DHT. The K_ds were calculated as described in Materials and Methods.

As expected, mutants containing the M107I replacement showed a modest increase in the K_d of DHT, yielding rabbit DHT binding affinity (Table 1). This difference might be the result of loss of a van der Waals contact of the S^δ of methionine in the DHT bound protein; whereas two van der Waals contacts between I107 and E₂ seem to persist when E₂ is bound (data not shown) and its K_d remains unchanged.

Interestingly, when the E₂-bound M107I structure is examined, F56 shifts but the distance between ring A and F56 does not change. M107 shows up as one of the larger main-

Fig. 4. Determination of DHT and E₂ equilibrium dissociation constants (K_ds) of the COS-expressed M107I/I138V/R140K/I141L mutant and plasma rabbit SBP by direct and competitive Scatchard analysis at pH 7.4 and 4°C. (A) M107I/I138V/R140K/I141L, (solid circles) SBP samples (2 nM) incubated with a range of 1–10 nM [1,2-³H]DHT; (open circles) SBP samples (2 nM) incubated with 25.33 nM radioinert E₂ and a range of 1–10 nM [1,2-³H]DHT. (B) Rabbit SBP, (solid circles) SBP samples (2 nM) incubated with a range of 1–10 nM [1,2-³H]DHT; (open circles) SBP samples (2 nM) incubated with 50.67 nM radioinert E₂ and a range of 1–10 nM [1,2-³H]DHT. The K_ds were calculated as described in Materials and Methods.

Fig. 5. Stereoscopic view of the steroid binding site of wild type human SBP bound with DHT (SBP, purple; DHT, yellow) and E₂ (SBP, green; E₂, red). The carbonyl oxygen of DHT forms a hydrogen bond with Ser42 OG at the bottom of the figure; whereas the hydroxyl oxygen in ring A of E₂ cannot because of the planarity of the now-aromatic ring A. The hydrogen-bonding network between Y57/D59/R140 is at the top of the figure. M107 lies on the far side of the steroid from the viewer.

chain, but not side-chain, shifts in all of the other comparisons. Because it is so closely packed with E₂/DHT, M107 is clearly quite sensitive to the steroid location, perhaps acting as an effective tumbler in the lock that holds the steroid, but repositioning itself so that the side-chain atoms remain close but the main-chain atoms move more, much as is the case for M139 (see Materials and Methods).

When E₂ is modeled into the binding site of the triple mutant, two changes take place. First there is a systematic and concerted shift of residues around the binding site, shifts which are expected to be more or less energetically equivalent. Second, there is a resultant increase in the distance of F56 from ring A of E₂ (Fig. 6B). In the cases of R140K and I141L alone (data not shown), the distance separating the closest C of F56 and C4 of ring A of E₂ was ~3.7Å, compared to ~3.3Å for the wild type. That distance becomes 4.1Å in the M107I/R140K/I141L mutant, which binds E₂ the least (Table 1).

The increase in separation of F56 from ring A of E₂ in the R140K mutant results from the concerted shift of the β sheet containing F56 that occurs when the two hydrogen bonds formed between R140 and D59 are reduced to one formed between Lys 140 and D59, along with concomitant repacking of the lysine side chain. The replacement of I141 by Leu apparently accomplishes a similar motion, but it is not clear why. When both mutations are present, both the systematic shift as well as about a 0.4 Å displacement of E₂ away from its position in the unmodified protein produce

the larger distance reflected in the poorer interactions between F56 and estradiol.

Discussion

The results presented here indicate that replacements at R140 and I141 with the corresponding rabbit SBP residues K and L, respectively, were sufficient to lower the E₂-binding affinity of human SBP without affecting the affinity of DHT. In addition; the data in Table 1 also confirm that replacing methionine with isoleucine at position 107 in human SBP reduces DHT-binding affinity to rabbit SBP level but has no effect on E₂-binding affinity (Petra et al. 2000). When this mutation work was near completion, the crystal structure of a truncated version of human SBP (the N-terminal domain from residues 13 to 188) bound with DHT was published (Grishkovskaya et al. 2000). The structure revealed a dimer having two steroid-binding sites located in each subunit 25 Å apart. The presence of two fully occupied and independent steroid-binding sites was surprising in view of the reported one mole ligand/dimer stoichiometry for the full-length native protein (Petra et al. 1986 and references cited therein). These authors suggest that high steroid concentration during crystallization may have saturated the second site by disrupting the allosteric transition that might normally block binding of the second ligand, but since the binding stoichiometry of the truncated dimer in solution is not known, it seems more likely that the nega-

Fig. 6. Stereoscopic view of (A) wild-type human SBP bound DHT (SBP, purple; DHT, yellow) compared with mutant M107I/R140K/I141L bound with DHT (SBP, green; HT, red), and (B) wild-type human SBP bound with E₂ (SBP, purple; E₂, yellow) compared with mutant M107I/R140K/I141L bound with E₂ (SBP, green; E₂, red). For clarity the hydrogen bonds are shown for only one molecule but exist for both in A. Note that the hydrogen bond S42 OG...O3 is present in both the unmutated and mutated molecules in A and absent for both in B. Although there is a small systematic shift of the chains visible in the mutated protein in A, it is larger in B, and there is a slight displacement of E₂ away from F56 as well. The hydrogen bond between K140 and D59 is not shown in B but can be seen to approximately replace the lower of the two hydrogen bonds shown for R140 to D59.

tive-binding cooperativity previously shown for the native protein (Namkung and Petra 1986; Petra et al. 1988) results from the as yet structurally uncharacterized C-terminal domain in the context of the entire SBP molecule. The latter could play a role in masking the second potential binding site in the opposite subunit once the first site is occupied. Hence, a crystal structure of the full-length native protein will have to be determined before we can fully understand the negative cooperativity aspect of the steroid-binding mechanism of human SBP.

The X-ray structure confirms steroid-binding site residues that had been previously identified, particularly M107 and M139 (Petra et al. 2000; Grenot et al. 1992; Sui et al. 1992); however, the side chains of R140 and I141 are not found to make contact with the ligand, thus precluding a direct interaction as the explanation for differences in E₂ affinity. To search for a structural explanation for the loss of E₂ binding in the absence of X-ray structures of the mutants, we carried out modeling studies. As shown in Figure 5, due to flattening of ring A, the H-bond at S42 is eliminated

when E₂ is modeled into the site, but the side chains of R140 and I141 are not altered with respect to their original positions. Notably, the H-bond network joining R140, D59, and Y57 is not disrupted, supporting our view that the 10-fold difference between E₂- and DHT-binding affinity of human SBP (Table 1) can be satisfactorily explained by elimination of the H-bond at S42. When either R140 or I141 is replaced with K and L, respectively, the DHT-binding affinity remains the same but the E₂ K_d increases about 4-fold and the phenyl ring of F56 and ring A of 17 β -estradiol move $\sim$ 0.6 to $\sim$ 0.7 Å farther apart (data not shown).

The loss of van der Waals interactions at F56 when E₂ is bound occurs as a result of a systematic concerted shift of residues 138–141 and 56–59 (compare Figs. 5 and 6B), because these two regions are hydrogen bonded to each other in a β -sheet. The shifts and lowered affinity of E₂ are the result of two factors: loss of one H-bond in the network among Y57/D59/R140 and changes in packing of E₂ primarily with F56. DHT does not change location in the triple mutant because of the strong hydrogen bond to S42. In human SBP, residue R140 forms two H-bonds to D59, but only one is formed when the residue is mutated to K. Moreover, D59 is still hydrogen bonded to Y57; so in order to form this new H-bond, the lysine atoms in residue 141 must translate farther down (in the orientation shown in Fig. 6), and this is apparently accommodated only by concomitant shifting of residue 140 and residues 56–69 that form main-chain hydrogen bonds to 140–141. The repacking of residues thus results in less optimal packing of F56 against E₂. The I141L mutation places an additional methyl group near E₂; why this also pushes residue 56 away from ring A of E₂ is less obvious. The I141L mutation has little effect on DHT, which cannot repack further without disturbing the S42 interaction. The triple mutant combines all these effects.

Our previous work has shown that the Y57F mutation in human SBP causes a modest decrease in binding affinity of DHT but has no effect on the binding of E₂ (Petra et al. 2000). This mutation would be predicted to cause a loss of one H-bond in the Y57/D59/R140 network, and therefore should cause an effect similar to the loss of a H-bond in the R140K mutation. However, when we modeled the Y57F mutation, we indeed found a similar concerted motion of residues 56–59 and 140–141, but F56 had adopted positions in both the unmutated and mutated forms that effectively yielded the same van der Waals interactions with E₂, explaining why the binding affinity of E₂ was unaffected (data not shown). This finding shows the validity of our modeling studies with the conclusion that the resultant relative movement of E₂ and F56 is what directly affects the affinity of E₂.

When both R140 and I141 were replaced by K and L, respectively, the E₂ affinity dropped to a level closer to that of rabbit SBP, and the distance separating the phenyl ring of

F56 and ring A of E₂ increased to $\sim$ 4.1 Å (Fig. 6B). We therefore conclude that the difference in estradiol-binding affinity between rabbit and human SBP is mainly due to the contribution of F56 to the hydrophobic interaction between the latter and ring A of E₂. In addition, we believe that the presence of K and L at the homologous positions 134 and 135 of rabbit SBP (Fig. 1; Griffin et al. 1989; Lee et al. 1997) places the conserved F50 (homologous to F56 in human SBP) far enough from ring A of E₂ to disallow optimal interaction. We would further expect that the 15-fold increase in the E₂ K_d of the M107I/I138V/R140K/I141L mutant is equivalent to a loss of about 1 kcal/mole of binding energy. That such small changes in structure are effective in changing binding energies is supported by a recent mutational study on the streptavidin-biotin complex, where a small structural change in the binding pocket increased the K_d 1000-fold and resulted in the loss of 4.3 kcal/mole of binding energy (Freitag et al. 1999). That small structural changes lead to significant changes in binding affinities makes sense in the evolution of the human SBP steroid-binding site. Because rabbit SBP binds only DHT with high affinity, the ability of the human protein to bind both DHT and E₂ with only a 10-fold difference in affinity may have been a more recent evolutionary event. The data presented here suggest that the mechanism for acquiring this new function involved the selection of structural changes outside the immediate environment of the steroid-binding site. Mutations of important residues such as M107, M139, S42, or D65 within the binding site would have been too drastic for human SBP to acquire the ability to bind E₂ without compromising the binding of DHT. Instead, by changing residues not directly involved in DHT binding, such as those at positions 140 and 141, it was possible to induce a subtle structural change at a distance from the steroid-binding site, resulting in the movement of an important side chain, the phenyl ring of F56, without disrupting other interactions. Such a mutational event could have established the new dual-binding specificity of the human protein. Taken together with a recent study on similar alterations near the active site of thymidylate synthase via random mutagenesis and selection of functional molecules with altered drug resistance (Landis et al. 1999), we believe that mutagenesis of next-nearest neighbors to a functional site could be part of the general mechanism for the evolution of altered specificities in pre-existing protein-active sites.

Availability of the M107I/I138V/R140K/I141L mutant will allow more detailed exploration of the proposed controversial role of SBP in the assisted diffusion of sex steroids into target cells. Because to date this is the only human SBP mutant found not to bind E₂ significantly, one could design cell experiments to explore whether or not SBP has an effect on the specific cell uptake of E₂ using wild-type human SBP as control. Unlike rabbit SBP, which is the only

other purified androgen-binding protein available for such experiments, the human M107I/I138V/R140K/I141L mutant would be expected to bind normally to the human SBP receptor; whereas the former may not. In this context, it might be useful to construct a mutant that binds E_2 normally but not DHT by keeping wild-type residues at positions 140 and 141 and changing nearby residues. This undertaking, however, is not likely to succeed because, according to the X-ray structure, the interactions near rings C and D of DHT (H-bonds at D65 and N82 and contributions of M107 and M139) are required for binding both steroids. It seems that the steroid-binding site of SBP was primarily designed to bind T (testosterone) and DHT and, according to the discussion presented above, it would appear that the ability to bind E_2 was superimposed later onto the original structural design. Therefore, attempts by site-directed mutagenesis to modify the steroid-binding site to accommodate only E_2 are likely to fail and result in the elimination of binding affinity for both steroids. Finally, identification of R140 and I141 as important factors for binding E_2 offers the possibility of designing inhibitors that can compete specifically for either E_2 or DHT, which in turn may have clinical applications since SBP is believed to play a role in signaling and/or in sex steroid hormone cell uptake by target cells.

Materials and methods

Materials

pcDNA3 was purchased from Invitrogen. QuickChange site-directed mutagenesis kits and Epicurian Coli supercompetent cells were purchased from Stratagene. The AmpliCycle sequencing kit was purchased from Perkin Elmer. 5α -dihydrotestosterone (DHT), testosterone (T), and 17β -estradiol (E_2) were purchased from Steraloids. DMEM, calf serum, AIM V Media, Lipofectamine 2000 Reagent, and Custom primers were ordered from Life Technologies. $[1,2-^3H]$ DHT (58.4 Ci/mM) and $[6,7-^3H]E_2$ (48 Ci/mM) were purchased from New England Nuclear. ELISA Kits for SBP determinations were provided by RADIM, and rabbit anti-goat IgG coupled to alkaline phosphatase used in Western blots was bought from Bio-Rad. Rabbit plasma SBP was obtained from Pel-Freez Biologicals. All other equipment and chemicals used in this work were described in cited publications from this laboratory.

DNA constructions, site-directed mutagenesis, and SBP expression

Full-length wild-type SBP cDNA (Hagen et al. 1992) was subcloned in pDNA3 and mutated with combinatorial sense and antisense oligonucleotides with the QuickChange procedure, using *Pfu* DNA polymerase. Mutated cDNAs were recovered by digesting the wild-type template strand with *Dpn* I, which targets the sequence 5'-G^mATC-3' (Nelson and McClelland 1992). The remaining single-stranded nicked plasmid was transformed into competent *Escherichia coli* and colonies were isolated for DNA purification and sequencing. cDNAs were transfected and expressed in COS-7 cells using Lipofectamine 2000 Reagent, as

described by the manufacturer, and cells were grown in serum-free AIM V medium for SBP expression.

Determination of equilibrium constants of dissociation

The total concentration of SBP in transfection media were determined by the charcoal assay with $[1,2-^3H]$ DHT (Sui et al., 1992). Equilibrium constants of DHT dissociation were determined directly by Scatchard analyses using SBP concentrations ranging from 1 to 2 nM and $[1,2-^3H]$ DHT from 1 to 10 nM. Equilibrium constants of E_2 dissociation were determined either directly using 1–10 nM range of $[6,7-^3H]E_2$ concentration or by competitive Scatchard analyses using a range of 1–10 nM $[1,2-^3H]$ DHT in the presence of radioinert E_2 and the equation $K_p = K_d (1 + [E_2]/K_i)$, where K_d = equilibrium constant of $[1,2-^3H]$ DHT dissociation, $K_p = K_d$ in the presence of radioinert E_2 , and K_i = equilibrium constant of E_2 dissociation (Mickelson and Petra 1978). Concentrations of radioinert E_2 used in the calculation of K_i by competitive Scatchard analyses are shown in the figure legends and Table I. It should be noted that the concentration of radioinert E_2 used for determining the E_2 K_d by competitive Scatchard analysis needs to be lower than 50 nM; otherwise, overestimated K_d values will be obtained at higher levels, as previously found (Sui et al. 1996).

Molecular modeling

Coordinates for the recently determined N-terminal domain (residues 13–188) of the sex-hormone-binding protein (Grishkovskaya et al. 2000) were obtained from the Protein Data Bank (code 1D2S; Berman et al. 2000). This 1.55 Å structure contains 5α -dihydrotestosterone (DHT) and alternate locations for 10 surface residues; for purposes of further computation only the A conformations were used. Estradiol (E_2) was constructed from the DHT coordinates in the 1D2S structure by removing the C19 methyl group, aromatizing ring A, and replacing the carbonyl oxygen at C3 with a hydroxyl group. To construct models to explore reasons for the differences in binding of DHT and E_2 to mutants, the following procedure was used. All atoms beyond 15 Å from the steroid atoms were held fixed. The steroid was removed and an energy minimization carried out on a Silicon Graphics O2 computer using the software suite Molecular Operating Environment (MOE; version 1999.05), available from the Chemical Computing Group (<http://www.chemcomp.com>). All minimizations energy parameters from the Koll94 set (Weiner et al. 1984) were used with default weights, except that planar restraints were weighted 100 times more. Hydrogen positions were calculated and chiral centers were restrained to their current value. The MOE energy minimization procedure carries out 100 steepest descent, 100 conjugate gradient and 200 truncated Newton iterations. The force on each atom was monitored visually on the graphics screen; when there was no large force visible, the change in energy between cycles was small and the gradient <0.2, the minimization was terminated manually. Starting with a minimized empty structure, each steroid was added back and protein plus steroid minimized to give the base structures to which mutants would be compared. Each mutant was made individually and atoms within 15 Å of the mutated site were minimized; then each steroid was added separately and minimized along with the protein to give the bound-mutant forms. The root mean square difference between main-chain atom positions of the X-ray structure and the minimized structure with DHT added back was ~0.36 Å, with the exception of H83, M139, and Ser-Gly 128–129. The X-ray structure does not contain coordinates for residues

130–135: This is perhaps why residues 128–129 differ. H83 is on the surface and away from regions of interest. M139 appears to differ more at the main chain than it does at the S^{δ} – C^{ϵ} atoms, which are packed up against the steroid. The root mean square differences between coordinate sets of mutants with the same steroid bound did not exceed 0.35 Å for main-chain atoms or 0.5 Å for side-chain atoms. The residues that change the root mean square difference from 0.35 Å to 0.5 Å are on the surface of the structure, where there are fewer restraints, suggesting that those changes may not represent functionally significant differences in the binding of the steroid.

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