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Localization of the steroid-binding site of the human sex steroid-binding protein of plasma (SBP or SHBG) by site-directed mutagenesis

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The amino-terminal region of the human sex steroid-binding protein of plasma (SBP or SHBG) containing K134 and M139 was found to represent part of the steroid-binding site. This was accomplished by constructing and expressing site-directed mutants having the following replacements: M139L, M139K, M139S, K134A, H235S, and Y57F. The results indicated that M139L and H235S were fully-active, K134A and Y57F were 50 and 67% active, M139K was 7% active, and M139S was inactive. These results support affinity-labeling data indicating that both K134 and M139 are located in or near the site, and suggest that Y57 may play a role in steroid binding. The fully active H235S mutant reveals that H235 is not involved in the steroid-binding process.

Sex steroid-binding protein; SBP; Sex hormone binding globulin, SHBG; Mammalian expression; Site-directed mutagenesis

1. INTRODUCTION

The human sex steroid-binding protein, SBP (or sex hormone binding globulin, SHBG) is a 93.4 kDa homodimeric glycoprotein that specifically binds testosterone (T) and 17β-estradiol (E_2) with high affinity in plasma, controls their metabolic clearance rates, and has been implicated in the endocytosis of sex steroid hormones into target cells (see [1] for a review). The human and rabbit proteins have been extensively characterized [2] and their amino acid sequences were determined [3,4]. The human SBP sequence agrees with that deduced from a full-length cDNA constructed from PCR fragments [1,5]. The oligosaccharide side-chains of hSBP are linked to T7, N351, and N367, and were found to play no role in steroid binding [6]. Human SBP has been expressed in BHK-21 and COS-7 cells [5] and in CHO cells [7]. This laboratory is interested in developing SBP as a protein model to understand the molecular basis of protein–steroid binding and the role of the protein in sex steroid hormone transport. Recently, the steroid-binding site of the human protein was tentatively identified in the region incorporating residues K134 and M139, as determined by affinity labeling with 5α-dihydrotestosterone derivatives [8,9]. In order to confirm this result, as well as testing other residues implicated in the steroid-binding process, we have mutated K134, M139, and other residues, and related the consequence of their replacements to the steroid-binding activity of the expressed mutated recombinant proteins.

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2. MATERIALS AND METHODS

A full-length hSBP cDNA was cloned in pBluescript II SK (Stratagene) as previously described [5]. Site-directed mutagenesis was carried out according to Kunkel et al. [10] using the Version 2 Muta-Gene Phagemid kit (Bio-Rad) with antisense mutated oligonucleotides synthesized on an Applied Biosystems model 380A DNA synthesizer. Mutated cDNAs were subcloned into pcDNA/Neo (Invitrogen) as previously described [5] and sites of mutations were confirmed by double-stranded DNA sequencing using the Sanger method [11]. Transfections of mutated pcDNAI/ Neo/SBP and wild-type into COS-7 cells were mediated by DEAE–dextran [12] along with control transfections with pcDNAI/Neo. Cells were grown in DMEM medium containing 5% calf serum. Media from transfected cells were collected 6 days after transfection and assayed by ELISA [13] and charcoal adsorption. The latter was carried out by incubating samples with a 3–4-fold molar excess of [^{3}H]DHT over SBP (± a 100-fold molar excess of radio-inert DHT) for 20 min at 25°C and 10 min in ice. The samples were added to a charcoal pellet prepared from a standard solution (0.5% Norit A, 0.05% dextran T-70, 0.1% gelatin) using twice the SBP sample volume. The mixture was mixed, left on ice for 10 min, and spun for 2 min at 14,000 rpm. Competition studies were carried out by incubating the medium with 10 nM [^{3}H]5α-DHT (5α-dihydrotestosterone) and a 100-fold molar excess of radio-inert DHT, T, E_2 (17β-estradiol), P (progesterone), and F (cortisol).

3. RESULTS AND DISCUSSION

The experiments presented here were designed to test whether or not K134, M139, H235 and Y57 play a role in the steroid-binding activity of SBP. A total of seven mutants were constructed and expressed: M139L, M139K, M139S, M139D, K134A, H235S, and Y57F. Fig. 1 shows portions of the DNA sequencing gels coding for the K134A and M139S replacements, along with the sequence of wild-type SBP encompassing these two residues. All other gels demonstrated that the proper mutations had been made (data not shown). Since pro-

tein stability is related to tight packing of amino acid side chains within the interior of proteins, where, in the case of SBP, the steroid-binding site is likely to be located, we chose to mutate with residues similar or smaller than the wild-type to minimize perturbation in internal packing which could result in loss of activity through undesirable conformation changes. The empty space arising from replacing with smaller residues would be expected to be filled through slight movements of surrounding side chains thus decreasing the chance of producing large non-specific conformation changes. Loss of activity in such cases is therefore more likely to reflect a loss of function by the elimination of a steroid-binding group. The M139L replacement matches as closely as possible the structure of methionine whereas those in the M139S, M139K, and M139D mutants depict changes in size and hydrophobicity. Removal of most of the lysine side chain in the K134A mutant and the imidazole ring in the H235S mutant were carried out to test the role of these residues previously implicated in steroid-binding [9,14]. Also included was a Y57F mutant to test a possible role for the sole phenolic group in SBP. Table I shows that major changes in the structure of the M139 side chain reduce the specific DHT-binding activity. As expected, a change to leucine has little effect on the activity, suggesting that a certain freedom of movement of the methionine side chain can be tolerated within the binding site. However, any decrease in size or hydrophobicity, as indicated by the serine and lysine replacements, produces a major reduction in activity. Also, elimination of most of the lysine side chain of K134 and the phenolic group of Y57 reduces the activity. The M139D mutant could not be tested because its expression in COS-7 cells was extremely low even after repeated transfections. Table I and the Scatchard plots in Fig. 2 also reveal that the apparent K_ds for both M139L and H235S are similar to wild-type SBP, while those for M139K, M139S, K134A, and Y57F mutants are markedly higher, as predicted from the specific activities shown in the Table. Since the rates of DHT dissociation for the M139K, M139S, K134A, and Y57F mutants are unknown, we must emphasize that the values of these K_ds may not reflect a true measure of the dissociation constants since the equilibrium concentration of bound DHT could have changed during the measurement if the rates of dissociation were high. Nonetheless, they are markedly different from those of the M139L and H235S mutants which bind DHT with the same affinity as the wild-type. The results therefore strongly indicate that the M139 side-chain contributes significant energy of binding to the steroid and thus resides within the binding site. We offer the following explanation for the activity loss of the M139K and M139S mutants: presence of the positive

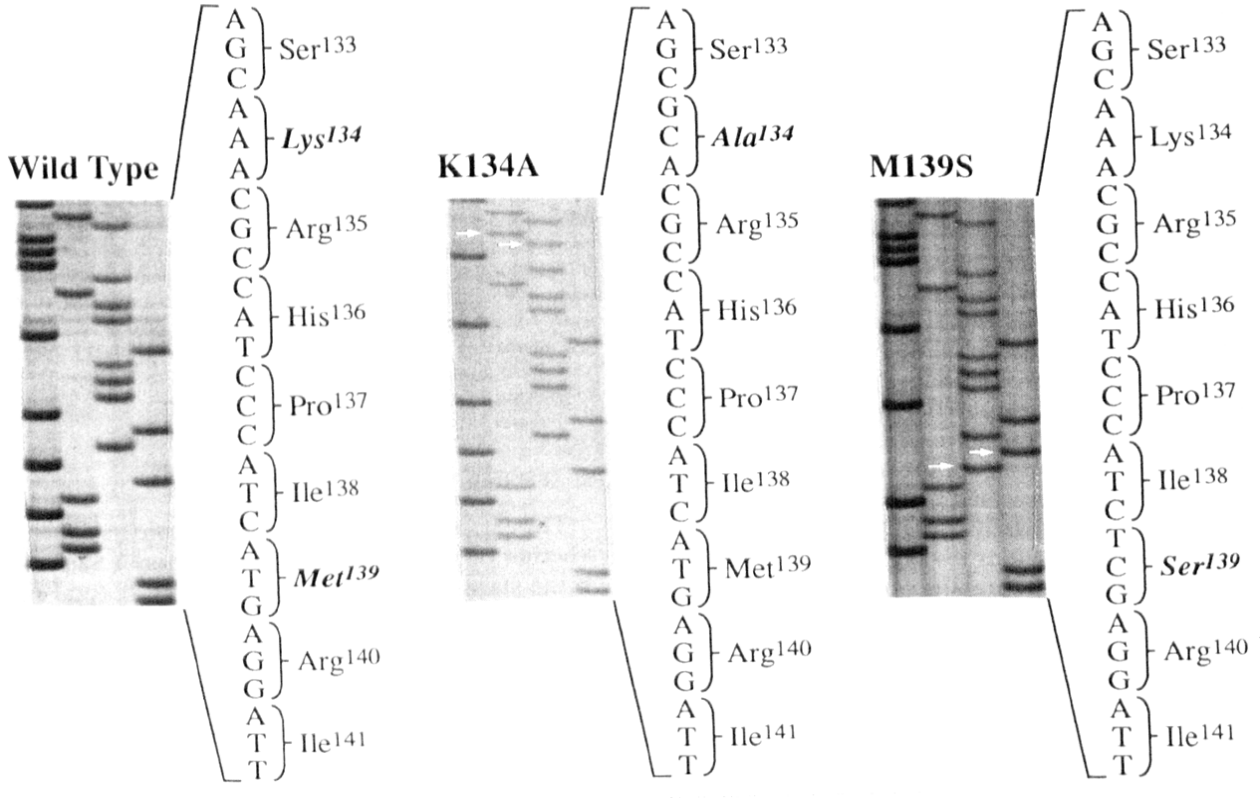

Fig. 1. DNA sequencing gels indicating portions of the SBP sequence incorporating K134 and M139. The arrows point to mutated nucleotides coding for the indicated amino acid replacements.

Table I

Steroid-binding properties and levels of wild-type and mutated SBPs secreted by transfected COS-7 cells

	DHT binding (nM)	ELISA (nM)	Specific activity (DHT binding/ELISA)	K_d (nM)
pcDNAI/Neo (control, no SBP)	0.30[b]	0[b]	0	0.76[c]
pcDNAI/Neo/SBP (wild-type)	1.86	2.48	0.75 (100%)	0.68
M139L	2.04	2.91	0.70 (93%)	0.74
M139K	0.11	2.35	0.05 (7%)	2.30
M139S	0	1.16	0 (0%)	1.10[c]
K134A	1.28	3.39	0.38 (50%)	2.11
H235S	2.32	2.68	0.87 (116%)	0.85
Y57F	1.40	2.80	0.50 (67%)	2.00

[a] These values represent specific binding measured by the charcoal assay at ligand saturation. Each value was corrected for non-specific binding by using a 100-fold molar excess of radio-inert DHT.

[b] This amount of DHT binding activity corresponds to the low concentrations of calf SBP in the culture medium (5% calf serum). The ELISA cannot detect calf SBP because the polyclonal antibodies raised against hSBP do not cross-react with it [16]. That amount of DHT binding activity was subtracted from all the values in the Table.

[c] The total DHT-binding activity of the M139S mutant was equal to that measured in the culture medium containing COS-7 cells transfected with control pcDNAI/Neo. These values therefore correspond to the K_d of calf SBP. The variation is within experimental errors.

charge in the former inhibits steroid binding due to repulsion by the positive charge of K139, while removal of the methyl and sulfur groups in the latter reduces hydrophobicity and may result in the partial collapse of the binding site. A recent report indicates that a M139W SBP mutant has a lower steroid-binding affinity than wild-type SBP [15]. Although that observation agrees with the conclusions presented here, one sole replacement by a larger residue, such as tryptophan, is difficult to interpret in terms of a structure–function relationship for the reasons stated above. Although a large conformation change may not necessarily occur in this case, the significant structural change at the immediate site of replacement is likely to be too drastic to interpret the resulting loss of activity solely on the basis of the elimination of the methionine side chain. Other replacements, particularly by smaller residues as done here, must be carried out before a conclusion can be made. Lastly, competition experiments with T, E_2, P, and F show that the active mutants have the same steroid-binding specificities as wild-type SBP (data not shown).

As shown in Table I and Fig. 2, expression of the K134A mutant, lacking most of the lysine side chain, produced a recombinant SBP that had lost about 50% of its DHT-binding activity and had a higher apparent K_d. Due to its proximity to M139, removal of the K134 side chain probably induced a conformation change in the steroid-binding site through the elimination of a stabilizing salt-bridge, as proposed recently [1,9]. In order to explain the complete loss of activity obtained in the specific alkylation of K134 by the affinity label, 17β-[([2-^{14}C]bromoacetyl)oxy]-5α-androstan-3-one [9], we propose that reaction by the bromomethyl ketone group of the label took place by extending to the outside of the site, about 6–8 Å from the C-17 of the steroid (the approximate length of the bromomethyl ketone group) where K134 is probably located. Covalent attachment of the label to K134 must have caused inactivation by blocking the site, thus preventing [^{3}H]DHT from binding during the assay. We conclude that K134 is probably located at the entrance of the site and is unlikely to contact the steroid as proposed earlier [1,9].

Mutation at H235 was carried out to resolve the existing controversy of whether or not this residue is present in the steroid-binding site [9,14]. Expression of a H235S mutant yielded a fully active recombinant protein, indicating that the imidazole ring of H235 is not present in the steroid-binding site or involved in stabilizing the

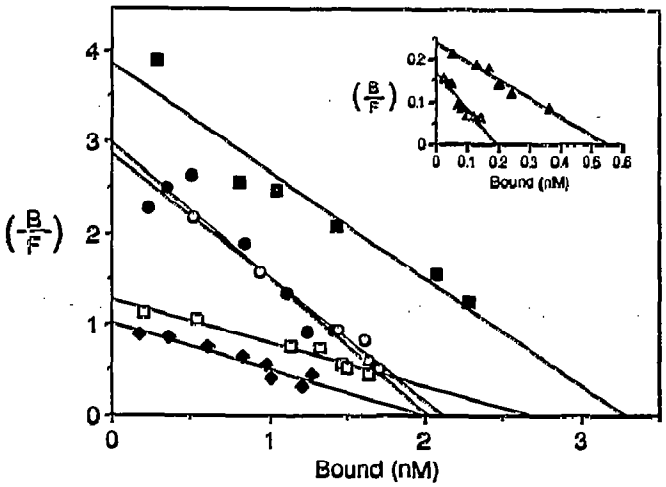

Fig. 2. Estimation of the equilibrium constants of dissociation of wild-type and mutant SBPs secreted by COS-7 cells using the charcoal method described in the text. The inset represents plots for the M139K and M139S mutants with expanded scale. (●) Wild-type; (■) H235S; (○) M139L; (□) K134A; (◆) Y57F; (▲) M139K; (△) M139S. The abscissa intercept for the M139L mutant is slightly greater than the wild-type. The K_ds calculated from these plots are listed in Table I.

site. In fact, the earlier claim for the alkylation of H235 by 17β-[([2-^{14}C]bromoacetyl)oxy]-5α-androstan-3-one was based on insufficient data (10 dpm over background, Table I of [14]). Removal of the phenolic group of Y57 lowers the activity, suggesting a possible role for this group in the steroid-binding activity, and indicating that the amino-terminal region of SBP containing Y57 may fold near the steroid-binding site. Other replacements at or near Y57 must be carried out to clarify the role of this residue in the steroid-binding process. In conclusion, the amino-terminal region containing K134 and M139 residues represents part of the steroid-binding site of human SBP. Other experiments are now in progress to expand on these observations with the ultimate goal of identifying all the residues involved in the steroid-binding process.

REFERENCES

[1] Petra, P.H. (1991) J. Steroid Biochem. Mol. Biol. 40, 735–753.
[2] Petra, P.H., Namkung, P.C., Senear, D.F., McCrae, D.A., Rousslang, K.W., Teller, D.C. and Ross, J.B.A. (1986) J. Ster. Biochem. 25, 191–200.
[3] Walsh, K.A., Titani, K., Kumar, S., Hayes, R. and Petra, P.H. (1986) Biochemistry 25, 7584–7590.
[4] Griffin, P.R., Kumar, S., Shabanowitz, J., Charbonneau, H., Namkung, P.C., Walsh, K.A., Hunt, D.F. and Petra, P.H. (1989) J. Biol. Chem. 264, 19066–19075.
[5] Hagen, F.S., Arguelles, C., Sui, L.M., Zhang, W., Seidel, P.R., Conroy, S.C. and Petra, P.H. (1992) FEBS Lett. 299, 23–27.
[6] Petra, P.H., Griffin, P.R., Yates III, J.R., Moore, K. and Zhang, W. (1992) Protein Sci. (in press).
[7] Bocchinfuso, W.P., Warmels-Rodenhiser, S. and Hammond, G.L. (1991) Mol. Endocrinol. 5, 1723–1729.
[8] Grenot, C., Montard, A., Blachere, T., Mappus, E. and Cuilleron, C.Y. (1988) C.R. Acad. Sci. Paris, t. 307, Serie III, p. 391–396.
[9] Namkung, P.C., Kumar, S., Walsh, K.A. and Petra, P.H. (1990) J. Biol. Chem. 265, 18345–18350.
[10] Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) Methods Enzymol. 154, 367–382.
[11] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[12] Ausubel, F.M., Brent, R. and Kingston, R.E. et al. (1990) in: Current Protocols in Molecular Biology, vol. 1, pp. 9.21–9.23, Wiley-Interscience, NY.
[13] Bordin, S., Torres, R. and Petra, P.H. (1982) J. Steroid Biochem. 17, 453–457.
[14] Khan, M.S. and Rosner, W. (1990) J. Biol. Chem. 265, 8431–8435.
[15] Bocchinfuso, W.P., Warmels-Rodenhiser, S. and Hammond, G.L. (1992) FEBS Lett. 301, 227–230.
[16] Petra, P.H. (1979) J. Steroid Biochem. 11, 245–252.