Specific Recognition of Androgens by Their Nuclear Receptor
A STRUCTURE-FUNCTION STUDY*

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Androgens, like progestins, are 3-ketosteroids with structural differences restricted to the 17β substituent in the steroid D-ring. To better understand the specific recognition of ligands by the human androgen receptor (hAR), a homology model of the ligand-binding domain (LBD) was constructed based on the progesterone receptor LBD crystal structure. Several mutants of residues potentially involved in the specific recognition of ligands in the hAR were constructed and tested for their ability to bind agonists. Their transactivation capacity in response to agonist (R1881) and antagonists (cyprotosterone acetate, CPA; cyproterone acetate; CPA, progesterone receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; ER, estrogen receptor; PR, progesterone receptor; GR, glucocorticoid receptor; CPA, cyproterone acetate; OH-Flu, hydroxyflutamide; R1881, methyltrienolone.

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The androgen receptor (AR) is a transcription factor that requires high affinity androgen binding to initiate a series of molecular events leading to the specific gene activation required for male sex development. This crucial role is demonstrated by the abundance of AR gene mutations identified in patients presenting with androgen insensitivity syndrome (1). This syndrome encompasses a wide spectrum of male pseudohermaphroditism ranging from complete androgen insensitivity to partial androgen insensitivity in subjects with female phenotype to partial androgen insensitivity syndrome in men with infertility and/or stigmata of undervirilization (2). Mutations have also been described in prostate cancer, and some of these alter the ligand-binding specificity, thereby inducing a putative AR activation by adrenal androgens (3) or the antiandrogens used during treatment (4).

AR is a member of the nuclear receptor (NR) family that includes receptors for steroid and thyroid hormones, vitamin D3 and retinoic acids, and numerous orphan receptors for which no ligands are known (5, 6). NRs are modular proteins that can be divided into separable domains with specific functions, such as ligand binding, dimerization, DNA binding, and transactivation. In the absence of ligand, the androgen receptor resides in the cytoplasm (7, 8). Hormone binding induces a transconformation of the receptor and allows its translocation into the nucleus where it initiates transcription through specific interactions with the transcription machinery (for review see Ref. 9). Recently, the crystal structures of unliganded and liganded NR ligand-binding domains (LBD) have been solved (10–17). These crystal structures reveal a triple-layered anti-parallel α-helical sandwich fold, with the major difference between the apo and holo states being the folding back of helix 12 toward the LBD core in the latter. This leads to a more compact structure of the liganded LBD (18).

Androgens, like progestins, glucocorticoids, and mineralocorticoids, are all 3-ketosteroids, with the structural differences between androgen and progestosterone restricted to the D-ring. In contrast, estrogens differ by their aromatic A-ring, with a phenolic hydroxyl at C3, but they exhibit a 17β-hydroxyl moiety in the D-ring similar to androgens. To better understand the interactions between the receptor and ligands, a three-dimensional guided mutagenesis approach was undertaken. For this purpose, three-dimensional models of the hAR-LBD complexes with several ligands are presented, which suggests new directions for drug design.

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1 The abbreviations used are: AR, androgen receptor; NR, nuclear receptor; LBD, ligand-binding domain; hAR, human androgen receptor; hRAR, human retinoic acid receptor; hER, human estrogen receptor; hPR, human progesterone receptor; hGR, human glucocorticoid receptor; MR, mineralocorticoid receptor; ER, estrogen receptor; PR, progesterone receptor; GR, glucocorticoid receptor; CPA, cyproterone acetate; OH-Flu, hydroxyflutamide; R1881, methyltrienolone.
Arg^{779}, Hia^{874}, and Thr^{877}. Substitutions of these residues could influence the interactions of C17 substituents with AR, resulting in the gain or loss of function. They were substituted by various amino acids by site-specific mutagenesis, and the resulting mutants were investigated for their ability to induce transcriptional activity in response to steroids with different C17 substituents. Furthermore, the repression exerted by antagonists on their transcriptional activity in response to agonist was also evaluated.

This study revealed that Arg^{779} is a crucial component of the architecture of the ligand-binding pocket and that Hia^{874} is not the anchoring site of androgens. In addition, the data showed that Thr^{877} contributes to the discrimination of 17β substituents together with Asn^{705}. The latter residue is more specifically involved in the anchoring of the 17β-hydroxy group of androgens and, surprisingly, the N705A hAR mutant exhibited PR-acquired properties. On the basis of the present work, general rules for the recognition of hAR agonist and antagonist ligands are proposed.

**EXPERIMENTAL PROCEDURES**

Sequence Alignment—The ClustalW 1.5 package (19) was used with default parameters to align the AR sequences with the other members of the steroid family and the hERs, human retinoid X receptor α, and hRARγ sequences for which three-dimensional crystal structures have been solved. First, the hGR, hPR, hAR, and MR sequences were aligned, and then hERa, human retinoid X receptor α, and hRARγ were added to the previous sequences in a profile alignment.

Model Building and Ligand Docking—A model of the hAR LBD was first generated by homology with hRARγ, and then as the structure became available, models were generated based on hERa and, finally, on hPR using the Modeller package (version 4.0, (20)). The homology model, discussed in this work, is based on the sequence alignment shown in Fig. 1A using the hPR crystal structure as a template. Ligands were positioned manually in the pocket using the probe-accessible and van der Waals volumes as guides (see “Determination of the Cavity Volumes”).

The cavity volume of the binding reaction. Cells were washed twice in phosphate-buffered saline and, 6 h later, transiently transfecting 200 ng of pCMVhAR or mutant AR DNA/well using the calcium phosphate co-precipitation method. Cells were maintained in 10% charcoal-treated steroid-depleted fetal calf serum-Dulbecco’s minimal essential medium for 48 h and then incubated for 2 h at 37 °C with increasing concentrations of [3H]R1881 from 0.2 to 4 nM, with or without a 100-fold molar excess of unlabeled R1881. Aliquots of total and free-labeled steroid were taken after the binding reaction. Cells were washed twice in phosphate-buffered saline and reextracted in 300 μl of 2% SDS, 10% glycerol, 2 mM EDTA, and 25 mM Tris, pH 7.8. Radioactivity was determined by scintillation counting, and specific binding was determined as the difference between total and nonspecific counts.

**RESULTS**

**Modeling of the AR Ligand-binding Domain—AR-LBD sequences from diverse organisms are highly conserved (over 80% sequence identity) compared with the other members of the steroid family (ARs share less than 20% sequence identity with ER, PR, GR, and MR).**

AR-LBD sequence alignment is presented in Fig. 1 together with some representative NR sequences. NRs share a common α-helical sandwich fold, composed predominantly of 12 helices, fairly conserved in the NR superfamily (18). Only the variable regions connecting the helices differ in length and composition; they adopt rather different spatial positions. Based on sequence alignment (Fig. 1), models of the hAR were constructed by homology using the hRARγ, hERα, and ultimately hPR crystal structures. Only the model based on the hPR crystal structure will be described. PR and AR exhibit 56% sequence identity, suggesting that a reasonable model can be built as shown by the backbone and side chain statistics obtained with Procheck.

The AR model revealed the ligand-binding pocket, which was further analyzed to identify the residues involved in the specific recognition of androgen ligands. It is lined by 19 amino acids that are mostly hydrophobic (Fig. 1B, green dots), with the exception of Asn^{705}, Gln^{711}, Arg^{759}, Gln^{791}, and Thr^{877}. It forms a pocket with an accessible volume (Connolly type volume) of 341 Å³, larger than the size of the ligands (testosterone, 252 Å³ and R1881, 270 Å³). The binding pocket is similar in size to that of the hERα-crystalline complex (cavity volume, 369 Å³ and estradiol volume, 236 Å³), and slightly smaller than that of the RARα-crystalline retinoic acid and PR-progesterone complexes (422 and 423 Å³ for the receptors and 278 and 301 Å³ for their respective ligands). We assumed that the A-ring anchoring pocket is shared between AR and PR. The receptor recognition motifs are located around the D-ring and involve loop 6–7, the N-terminal part of helix 11, and loop 11–12. The following residues, Asn^{705}, Arg^{759}, Hia^{874}, and Thr^{877}, belong to these segments and thus may participate in the specific recognition of androgens. We therefore constructed several mutants of these residues (N705A, R779A, R779S, R779Q, H874A, and T877C) and tested their influence on the D-ring anchoring for various AR and PR agonist or antagonist ligands (Fig. 2). The Arg^{729} was selected on the basis of the RARγ-generated AR model in which loop 6–7 lines the ligand-binding pocket, and this arginine points inside the binding pocket. This loop moves more
FIG. 1. Sequence alignment and homology model of the hAR-LBD. A, the alignment includes AR sequences from numerous organisms and also human GR, human MR, and human PR. The organism abbreviations are: hs, Homo sapiens; mm, Mus musculus; rn, Rattus norvegicus; sc, Serinus canaria; oc, Oryctolagus cuniculus (rabbit); xl, Xenopus laevis. The sequences of the human ERα, human RARγ, and human retinoid X receptor α for which crystal structures have been determined are also included. The sequence numbering is given for the hAR (top) and the hERα (bottom), respectively. Identical residues in the whole alignment are highlighted in yellow. Conserved residues among AR sequences are boxed in blue. Highly conserved residues between hAR, hMR, hGR, and hPR are boxed in green. Residues lining the ligand-binding cavity (at 4.5 Å from the ligand) of the hERα and hAR are represented as green and blue dots, respectively. B, homology model. Schematic drawing showing the overall fold of the hAR-LBD complexed with R1881. Residues in the vicinity of the ligand (4.5 Å) are depicted by gray-shaded and red spheres for hydrophobic and polar residues, respectively.
than 3 Å away, toward the surface in the PR-based AR model, and thus the Arg<sup>779</sup> side chain resides on the surface of the protein in this model. Located in helix 11, His<sup>874</sup> is reminiscent of His<sup>524</sup> in hER<sub>a</sub>, which is involved in a hydrogen bond with the 17β-hydroxyl moiety. Asn<sup>705</sup> and Thr<sup>877</sup> were selected on the basis of their potential implications in the D-ring anchoring highlighted by the three-dimensional model. Note that the residue at the position equivalent to Thr<sup>877</sup> in hAR is a cysteine residue in other steroid members (PR, GR, and MR).

**Ligand Binding to Mutant hARs—** COS-7 cells transfected with wild type or mutant AR expression vectors were incubated with varying concentrations of [3H]R1881 or [3H]R1881 plus a 100-fold excess of unlabeled R1881. The level of AR-bound R1881 was determined, and Scatchard analyses were performed. The <i>K<sub>d</i></i> and <i>B<sub>max</sub></i> values are reported in Table I. R1881 bound to the wild type hAR with an affinity of 0.55 ± 0.25 nM (n = 4), which is in the range of those previously reported (4–27). The T877C mutant displayed a lower affinity for R1881 with a <i>K<sub>d</i></i> of 2.0 ± 0.42 nM, in agreement with Ris-Stalpers et al. (27). The substitution of His<sup>874</sup> by alanine did not significantly alter the ligand-binding capacity (Table I). In our experimental conditions, using up to 10<sup>-7</sup> M radioactive R1881, we were unable to demonstrate steroid binding to the N705A and the three Arg<sup>779</sup> mutant hARs.

**Transactivation of Mutant hARs—** We tested the ability of the mutant ARs to activate transcription in response to R1881 (Fig. 3A), as well as various steroid compounds, namely progesterone (Fig. 3B), a synthetic progestagen R5020 (Fig. 3C), and cyproterone acetate (CPA), an antiandrogen with partial agonistic activity (28). Transcriptional activity was measured in CV1 cells transfected with an AR expression vector and a murine mammary tumor virus-Luc reporter gene. Activity was measured over a range of ligand concentrations and was expressed relative to the wild type AR activity in response to 10<sup>-9</sup> M R1881 (1076–1300-fold stimulation according to experiments), which was set at 100%. Substitution of His<sup>874</sup> by alanine induced a slight right-shifted dose-response curve, but maximal transcriptional capacity and the ED<sub>50</sub> value were similar to those of the wild type AR (ED<sub>50</sub> = 7.10<sup>−11</sup> versus 3.10<sup>−11</sup> for wild type AR). The T877C mutant hAR exhibited a right shift in the dose-response curve, nearly one order of magnitude (−3.10<sup>−11</sup>), with a reduced maximal level of transcriptional activity. The substitution of Asn<sup>705</sup> by alanine induced a larger shift toward higher concentrations, with an
Specific Recognition of Ligands by AR

Fig. 2. Chemical structure of ligands. hAR agonist (R1881 and testosterone), hPR agonist (progesterone and R5020), hAR antagonist (CPA, OH-Flu, and ICI176344 (Casodex)) and hPR antagonist (RU486) ligands are shown.

TABLE I
Equilibrium dissociation constant, $K_d$ (nM) and maximal binding capacity

|             | $K_d$  | $B_{max}$ (fmol/mg of protein) |
|-------------|--------|-------------------------------|
| Wild type   | 0.55 ± 0.25 | 1100 ± 214                   |
| N705A       | n.c.   | n.c.                          |
| R779Q       | n.c.   | n.c.                          |
| R779S       | n.c.   | n.c.                          |
| H874A       | 0.80 ± 0.33 | 1460 ± 250                   |
| T877C       | 2.0 ± 0.42  | 1530 ± 272                   |

ED$_{50}$ value of −1 μM, together with a preserved maximal transactivation capacity. No R1881-mediated transactivation function was detectable for the three Arg substitutions, even at micromolar concentrations. Western blot analysis of these receptors demonstrated that they were correctly expressed, indicating that their lack of activity was not attributable to improper expression (data not shown).

The R5020-mediated transactivation function was very low for wild type hAR and undetectable for H874A. The substitution of Thr$^{877}$ by cysteine resulted in an activation of 25% of the maximal activation observed for wild type hAR in response to R1881. In the case of N705A, mutant transcriptional activation in response to R5020 reached nearly 100%. These observations were even more surprising with progesterone (Fig. 3B). H874A hAR still failed to transactivate the reporter gene, whereas wild type and T877C hARs displayed, respectively, 25 and 30% activation at the higher concentration tested. In the same experiments, progesterone increased the luciferase activity of N705A hAR in a dose-dependent function very similar to that of wild type hPR, with a shift toward higher concentrations restricted to less than one order of magnitude.

The antiandrogen CPA possesses an A-ring and C17 substituents that differ significantly from R1881 (Fig. 2); nevertheless, it displayed partial agonist properties on the wild type hAR. Its ability to induce transactivation by H874A and N705A hAR was extremely low even at concentration in the micromolar range, in contrast to the wild type and T877C (respectively, 42 and 40% of $10^{-9}$ M R1881-induced activity). None of the tested substitutions improved transactivation efficiency in response to estradiol, RU486, or two nonsteroidal antiandrogens (hydroxyflutamide (OH-Flu) and ICI 176344 (Casodex)).

Antagonistic Properties—The extremely low CPA-induced transcriptional activity of H874A and N705A hARs may be because of an inability of the mutant receptor to bind CPA. To check this hypothesis, we evaluated the antagonism exerted by various concentrations of CPA on transactivation activity of both mutant hARs. We observed a clear antagonistic activity of CPA with H874A, even more potent than with wild type hAR, which clearly demonstrates that the mutant receptor binds CPA (Fig. 4A). Because N705A could not transactivate in response to R1881, we evaluated this antagonism on progesterone-induced transactivation activity (Fig. 4B). This was also performed with various other antagonists, RU486 and two nonsteroidal antiandrogens (OH-Flu and ICI176344). The two nonsteroidal antiandrogens exerted extremely low antagonistic properties on the N705A hAR mutant when compared with wild type androgen and progesterone receptor. In contrast, CPA and RU486 were still able to repress transcriptional activity of this mutant hAR. This strongly suggests the crucial role of the Asn$^{705}$ residue in the anchoring of the nonsteroidal antiandrogens.

Agonist Ligand Docking—The ligands depicted in Fig. 2 were docked in the light of the experimental data and according to the PR crystal structure in a complex with progesterone. Testosterone and R1881 were docked by superimposing the ligands on progesterone, followed by a few steps of minimization while keeping the backbone fixed. The binding pocket residues around the A-ring are rather conserved among the steroid nuclear receptors, especially the Gln$^{711}$ in the middle of H3 (Gln$^{725}$ in hPR and Glu$^{353}$ in hER) and Arg$^{752}$ (Arg$^{766}$ in hPR and Arg$^{394}$ in hER) at the end of H5, which, together with a water molecule, forms hydrogen bonds with the keto moiety in position 3.

The D-ring in testosterone and R1881 forms a hydrogen bond between the 17β-hydroxyl group and the carbonyl group of the Asn$^{705}$ side chain at 3.1 Å (Fig. 5). This asparagine is a well conserved residue in steroid receptors, which suggests its importance. The 17α-methyl group of R1881 is in van der Waals contact with Leu$^{704}$ and Leu$^{701}$. Close to these residues is a methionine (Met$^{760}$) that is conserved in AR, ER, GR, and MR, but there is a phenylalanine in PR, suggesting that a substituent at position 17α may be accommodated by the former receptors (e.g., 17α-ethynyl estradiol). T877 is just above the 17β position in the D-ring. The delta methyl group is oriented in the same direction as the sulfhydryl group in the cysteine residue (Cys$^{891}$) of PR, pointing away from the 17β-OH. Thr$^{877}$ has its...
hydroxyl group hydrogen-bonded to the backbone carbonyl of Leu873, and in this orientation the same group restricts the space around the 16β position. It is important to note that this moiety is rather distant from 17β-OH and unlikely to form a hydrogen bond.

**Antagonist Ligand Docking**—With these data in mind, the antagonist ligands (CPA, OH-Flu, and ICI176344) were docked. CPA was shifted and rotated in the cavity when compared with the other ligands. It exhibited a highly bent conformation because of the presence of a cyclopropyl ring in the

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**Fig. 3.** Transactivation profiles for wild type (WT) androgen receptor and mutants ARs in response to R1881 (A), progesterone (B), and R5020 (C). CV1 cells were cotransfected with murine mammary tumor virus-luc reporter, pCMVβ-Gal internal control plasmid, and the indicated AR expression plasmid. Transfected cells were then treated with R1881, R5020, or progesterone. Luciferase activities were normalized to β-galactosidase activity and are expressed relative to the wild type AR activity with $10^{-9}$ M R1881 (1076–1300-fold stimulation in different experiments), which is set at 100%. The values represent the mean S.D. from three or more experiments. For some values, error bars are too small to be seen.
A-ring. This movement most likely enables CPA to escape steric contacts with H3 around the cyclopropyl ring (Fig. 5) and Phe764 in the β-sheet region. The acetate moiety in 17α points toward Met780, a phenylalanine in PR, with its keto group involved in a hydrogen bond with the nearby Gln783.

OH-Flu was docked with the 2-hydroxyl moiety forming a hydrogen bond with the carbonyl group of the Asn705 side chain. The importance of this hydroxyl group has already been demonstrated (29), and upon substitution of Asn705 by alanine the action of OH-Flu is severely hampered, as previously shown (see “Antagonistic Properties” and Fig. 4). In this position, the carbonyl group of the amide linker is pointing between H7 and H10, away from H3, with the nitrogen atom superimposed on position 1 of the A-ring. The trifluoromethyl group is pointing in between the two methionine residues (Met745 and Met749). OH-Flu is a rather small ligand, and in this orientation, it

![Figure 4](#)

**Fig. 4.** A, repression of R1881-induced transactivation activity by CPA on wild type AR and mutant ARs. CV1 cells were transfected and processed for analysis of reporter gene activity exactly as described in the legend to Fig. 3, except that they were treated with R1881 10⁻¹⁰ M and various concentrations of CPA or vehicle. Luciferase activities were normalized to β-galactosidase activity and are expressed relative to the AR activity with 10⁻⁹ M R1881, which is set at 100% for each receptor. Values represent the mean S.D. from two or more experiments. For some values, error bars are too small to be seen. B, repression of R1881- or progesterone-induced transactivation activity by CPA, RU486, OH-Flu, or ICI 176344 (Casodex) of wild type (WT), N705A hAR, and wild type hPR. CV1 cells were transfected and processed for analysis of reporter gene activity exactly as described in the legend of Fig. 3, except that they were treated with R1881 10⁻¹⁰ M, progesterone 10⁻⁷ M, or progesterone 10⁻⁸ M, respectively, and various concentrations of antagonists. Luciferase activities were normalized to β-galactosidase activity and are expressed relative to luciferase activity in absence of antagonist, which is set at 100% for each receptor. The values represent the mean S.D. from three or more experiments.
makes no van der Waals contacts with residues that belong to the loop 11–12 and is thus unable to stabilize this flexible region of the ligand-binding pocket.

The “A-ring” region of ICI176344 was oriented similarly to OH-Flu but the anchoring of the D-ring differed significantly. In the active R conformation, the hydroxyl group of ICI176344 forms a hydrogen bond with Gln783. In this orientation, the aromatic ring, harboring the sulfonyl group, pushes away loop 11–12, resulting in an inappropriate folding. The sulfonyl moiety is pointing in between the C terminus of H11 and the N terminus of H7, suggesting that some nearby charged residues on the surface could stabilize this polar group.

RU486 was oriented with its steroid skeleton similar to that observed in the progesterone complex. In this position, its bulky substituent destabilizes H12 much the same as that noted in the crystal structure of Erα complexed with Raloxifene (13).

### DISCUSSION

The resolution of the crystal structures of the hErα-LBD and hPR-LBD, together with the structures of the evolutionarily more distant members of the NR family (RAR, human retinoid X receptor, peroxisome proliferator-activated receptor, and thyroid hormone receptor) (30), provide exceptional guidelines to model and analyze the hAR ligand-binding domain.

**A-ring Anchoring of Androgens**

The two steroid structures are closely related to the AR. They reveal the amino acids lining the ligand-binding cavity and unambiguously demonstrate the anchoring of the A-ring by two highly conserved residues in the steroid family (AR, GR, MR, and PR; Gln711 and Arg752 as in hAR, Fig. 1) that form a hydrogen bond network with the 3-keto moiety of their cognate ligand. These anchoring sites were recently confirmed for the hMR (31), and a similar network of hydrogen bonds is expected in AR because of the conservation of these amino acids. Clinical evidence of the implication of these residues in such a network is revealed by the two naturally occurring mutations identified in androgen insensitivity (R752Q (32), the testicular feminization mutation in rat (33), and Q711E, observed in two teenaged 46,XY sisters presenting with female phenotype and clitoromegaly).2 Furthermore, among the hydrophobic side chain in the vicinity of the steroid A-ring, mainly Met749 in H5 (close to the position four of the A-ring) differs compared with other steroid members. The presence in AR of a methionine residue, with a more flexible side chain, as compared with a leucine in the others members, may explain why more “exotic” A-rings have been observed among AR ligands (e.g. OH-Flu, ICI176344, or quinoline derivatives (34)). A second nearby methionine residue, Met745 in hAR, conserved in AR, PR, GR, but not in MR (Ser810 of hMR), and ER (Leu387 of hERα), may also contribute to this effect.

**D-ring Anchoring of Androgens**

The D-ring anchoring in the ligand-binding pocket is difficult to rationalize based solely on the model. This is especially because of the highly flexible loop 6–7, which adopts a rather different conformation when the ER and PR structures are compared with the RAR crystal structure. In the latter, the loop is part of the ligand-binding pocket, whereas in the former, this region moves some 3 Å toward the surface of the protein. This movement of loop 6–7 is concomitant to a bending of H3, resulting in a binding pocket that differs greatly from one receptor to another despite a similar fold. This is highlighted by the location of Arg779, which is part of the ligand-binding pocket in the RAR-based model but on the surface of the PR-based one.

**Role of Arg779 in the Architecture of the LBD**

The mutations

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2 N. Poujol, S. Lumbroso, D. and C. Sultan, unpublished data.
of Arg779 to alanine, serine, or glutamine were rather surprising, as they completely abrogated the ligand-binding and transactivation capacity of the receptor. They led to the same characteristics as those of the natural mutation (R779W) described in several unrelated patients presenting with complete androgen insensitivity syndrome (35). The fact that all the substitutions tested resulted in a total loss of activity, just the same as the natural mutation, suggests that Arg779 plays a crucial role in the architecture of the LBD, rather than only in the anchoring of the 17b-OH group. Such a drastic effect was also observed in the human PR where a tyrosine residue located at the surface was changed into phenylalanine, a residue observed in other PR species. This substitution resulted in a complete loss of ligand binding (36). The sequence-structure analysis of the environment of this residue revealed that the tyrosine formed two hydrogen bonds, whereas in the species exhibiting a phenylalanine, these polar interactions were replaced by hydrophobic contacts. A similar scenario could well explain the importance of the arginine side chain, where the charge and hydrophobic tail are of importance to maintain the architecture of the ligand-binding pocket.

His874 and T877C Are Not Hydrogen-bonded with the 17b-OH Group of Androgens—Substitution of His874 by alanine did not result in a significant loss of androgen binding, thus revealing that this residue is not involved in the anchoring site of the 17b-OH group of androgen ligands. This is in contrast to the role played by a similar histidine in the EREstradiol complex (13). His874 is located rather distantly from the ligand cavity but is in the vicinity of the C-terminal end of helix 12. The change to an alanine residue, with a shorter side chain, most likely destabilizes the positioning of H12 and results in a hAR receptor with a barely altered transactivation capacity in response to R1881. Ligands with bulky substituents in position 17, like CPA, which more efficiently antagonized R1881 action and became a true antagonist on the mutant receptor (Fig. 4A), revealed the importance of His874. Altogether, these data suggest that such ligands are too cumbersome for the hAR-ligand-binding pocket mutant. In particular, CPA adopts a bent conformation with its 17b chain directed toward loop 11–12, and thus it hampers the correct positioning of H12. In contrast, R1881, which bears a smaller C17 substituent (Fig. 2), is only marginally affected by this mutation, suggesting a perfect fit between the ligand and the binding pocket.

T877C Contributes to the Discrimination of the 17b Substituents—From the model, Thr877 is rather distant from the 17b-hydroxyl group (more than 4.0 Å) and restricts the space around the 16b position. Only a hydrogen bond with the backbone carbonyl of Leu873 is observed. The in vitro investigations are in agreement with these observations. The T877C mutant bound R1881 only 4-fold less efficiently than the wild type receptor, which is not in favor of a lost hydrogen bond between Thr877 and the 17b-hydroxyl group. Despite this, its transcriptional capacity was greatly affected and could not be restored even with high doses of ligand (10−5 M), suggesting that this substitution induces an alteration in the conformation of the LBD that partially hampers co-activator recruitment.  

Asn705 Is Essential for Anchoring AR Ligands—The N705A mutant showed a marked decrease in ligand-binding affinity, resulting in a drastic shift of the transactivation curve (four orders of magnitude, Fig. 3A). At a high dose the full transactivation capacity could be attained. Note that this mutant is more specifically activated by progestagens (Fig. 3, B and C). The Asn705 residue is most likely involved in the stabilization of loop 11–12 by the side chain NH2 moiety. An equivalent asparagine in the PR crystal structure has been shown to form a hydrogen bond with the carbonyl moiety of the backbone of Glu904 (Asp890 in hAR). The carbonyl group of Asn705 is implicated in a hydrogen bond with the hydroxyl moiety of R1881, OH-Flu, and ICI176344. Thus, the mutation to alanine creates some extra space in the binding cavity and removes a potential hydrogen bond acceptor. This explains the alteration in androgen binding, the shift of transactivation in response to R1881, and the marked decrease in the antagonistic activity of nonsteroidal antiandrogens.

N705A: an hAR Mutant That Acquires PR Properties

The N705A hAR mutant is unable to bind R1881 with high affinity, and micromolar concentration results in half the transactivation capacity of wild type hAR. Surprisingly, this hAR mutant was activated by progestagens similarly to that occurring in PR. These PR-acquired properties are puzzling because this asparagine is rather conserved among steroid receptors, and a similar mutation in PR affects agonist binding only marginally (26). In contrast, its critical role for agonist binding to the MR, which harbors a 21-hydroxyl moiety, has been shown (31) together with the absence of consequences for antagonists which lack such a substituent. The Asn705 residue faces Leu880, a rather bulky residue compared with the conserved threonine in the other steroid members (GR, PR, and MR). Androgens and progestagens differ mainly in the 17b substituent, where a hydroxyl group is replaced by an acetyl one (Fig. 2). The N705A mutation disrupts a network of hydrogen bonds and generates a hollow where the 17b substituents of progesterone or R5020 snugly fit. Both progestagens are able to activate the mutant receptor to near wild type PR levels (Fig. 3, B and C), despite a shift in ED50, suggesting that the mutation is sufficient to stabilize H12 in a position suitable for co-activator recruitment.

The conservation of this asparagine residue among the steroid receptors suggests that it is not involved in the specific discrimination between androgens and progestagens. Other differing residues in the binding cavity, such as Thr877 and Leu880, most likely exert such a discriminating effect. Both side chains surround the D-ring and restrict the bulkiness of the 17b extension of the ligands.

Antagonist Ligand Docking

The present results also highlight the crucial role of Asn705 in the antagonism capacities of nonsteroidal antiandrogens. OH-Flu and ICI176344 were less effective on this mutant receptor even at micromolar concentrations. In contrast, the steroidal antiandrogens CPA and RU486 prevented agonist-induced transactivation in the wild type AR, the wild type PR, and AR-N705A (Fig. 4B). In vitro investigations and three-dimensional AR models strongly suggest that the substitution of Asn705 by alanine results in a loss of contacts (hydrogen bond and/or van der Waals interactions) with the nonsteroidal antiandrogens that hamper their action.

Conversely, such contacts are less critical for the bulkier CPA and RU486, which exert their action on the mutant AR much as they do on wild type AR. Altogether, our results highlight the crucial role of this asparagine in defining the size and shape of the ligand-binding pocket that is essential for the specificity of transcriptional activation in the androgen receptor. Thus, the future design of agonists and antagonists of AR should introduce substituents able to contact this asparagine.

Conclusion

We constructed a homology model of the hAR ligand-binding domain using the hPR crystal structure. The analysis of the putative AR ligand-binding cavity and docking of high affinity ligands led to the identification of residues involved in AR-
specific D-ring anchoring. Some of them constitute potential interaction sites with AR ligands and can be exploited in the design of new AR drugs. Mutation of selected residues confirmed their role in ligand binding. In particular, a single mutation, N705A, leads to an AR chimeric receptor that exhibits PR-acquired properties. Furthermore, nonsteroidal antagonists (OH-flu and IC1176344, Fig. 2) are unable to antagonize agonist-induced transcriptional activity for this mutant, whereas wild type AR and PR do, revealing the crucial importance of this residue in the anchoring of antagonists. It remains to be determined whether other substitutions of Asn705, or of other residues in the vicinity of the D-ring, would induce similar altered ligand specificities. The importance of such mutations is buttressed by the existence of a natural mutant, N705S, that has already been identified in a patient with androgen insensitivity syndrome (37). One could envisage the use of progesterone or its derivatives to induce virilization in such patients.

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