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*Articles*

# Participation of Critical Residues from the Extreme C-Terminal End of the Human Androgen Receptor in the Ligand Binding Function†

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ABSTRACT: A short C-terminal end is present at the end of the human androgen receptor (hAR) similar to that of other steroid receptors. It is located directly after helix 12 of the ligand binding domain and has never been described as being part of the hydrophobic binding pocket. Although some fragmentary data have indicated the involvement of this region in ligand binding, its precise function still remains unclear. To gain deeper insight into the role of the hAR extreme C-terminal end, an extensive mutational analysis was carried out by using site-directed mutagenesis and alanine scanning over the 13-residue C-terminal end region. Both ligand binding and transcriptional activity were tested with each mutant. Our study demonstrates the participation of almost all of the amino acids in this region for the ligand binding function and consequently for the transcriptional activity. A conformational study by limited proteolysis was performed with the mutants that most affected the affinity of the receptor. It was remarkable that the mutants with a low binding affinity adopted an inactive conformation and were either less or not able to undergo a following conformational change to provide the active form of the receptor. Our results demonstrate the importance of hydrophobicity for the function of the C-terminal end with residues located at very precise positions. Especially, both hydrophobicity and aromaticity on position 916 are critical for providing the correct ligand binding conformation of the receptor. Furthermore, this study highlights essential criteria regarding the C-terminal amino acids which could be applied to other steroid receptors.

As is the case with most nuclear receptors, steroid receptors are hormone-regulated gene transcription factors which exert their actions after binding to hormones (for reviews, see refs *1*−*4*). Binding occurs through specific discontinuous sequences constituting the ligand binding hydrophobic pocket located within the 250 amino acid C-terminal part of the receptor—the ligand binding domain (LBD).[1] Since the crystal structure of several nuclear receptor LBDs has now been resolved, some progress has been made in describing the ligand binding hydrophobic pocket involved in the hormone recognition (*5*−*10*). However, not all of the precise sequences and mechanisms have been conclusively established. In particular, in the case of the steroid receptor subfamily including the androgen receptor (AR), the gluco-

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[1] Abbreviations: hAR, human androgen receptor; hGR, human glucocorticoid receptor; mGR, mouse glucocorticoid receptor; hMR, human mineralocorticoid receptor; hPR, human progesterone receptor; RAR, retinoic acid receptor; RXR, 9-*cis*-retinoic acid receptor; LBD, ligand binding domain; wt, wild type; AIS, androgen insensitivity syndrome; CAIS, complete androgen insensitivity syndrome; PAIS, partial androgen insensitivity syndrome.

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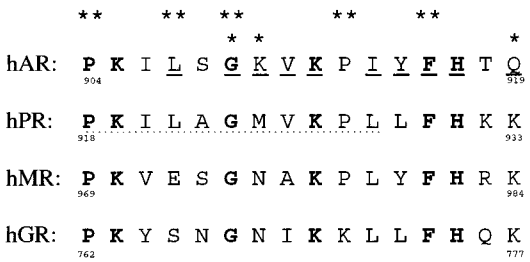


FIGURE 1: Amino acid sequence alignment of the C-terminal end from the hAR and other steroid receptors. Conserved residues within the steroid receptors are indicated by boldface letters. Underlined residues in the hAR sequence are the amino acids which have been mutated in this study. Mutations of the amino acids of the hAR indicated by two stars are associated to AIS (*16*, *17*). The positions associated to somatic mutations observed in cases of prostate cancers have been indicated by one star (*16*, *17*). In the sequence of the hPR, underdotted residues correspond to the sequence associated to the repressor function of hPR (*13*).

corticoid receptor (GR), the mineralocorticoid receptor (MR), and the progesterone receptor (PR), there is a short C-terminal sequence at the very end of the LBD (Figure 1) which has never been associated to the hydrophobic pocket although its partial or complete deletion abrogates agonist binding (*11*−*15*).

In the case of the human androgen receptor (hAR), it has been previously demonstrated that the truncation of the last 12 C-terminal amino acid residues (amino acid residues L907−Q919) abolishes hormone binding with both agonists and antagonists, except for the specific case of RU486 (*11*, *12*). Moreover, mutations implicated in abnormalities in ligand binding and receptor function have been found in the C-terminal end of the hAR, which lead to androgen insensitivity syndrome (either partial, PAIS, or complete, CAIS; for reviews, see refs *16* and *17*; for specific studies in the C-terminal end, see refs *18*−*21*). Some somatic mutations in the same region have also been described in metastatic prostate cancer samples (*22*−*24*).

Although deletion studies have demonstrated the clear role of the C-terminal end of steroid receptors on hormone binding (*11*−*15*), no extensive study has ever before been undertaken in order to elucidate which amino acid residue-(s) could be responsible for that function. Only a few studies in the case of mouse GR (mGR) and hMR have been performed on the position corresponding to the fourth amino acid residue before the end of the receptor, a conserved phenylalanine in all steroid receptors (Figure 1). When this position was mutated, the ligand binding was significantly decreased toward both agonists and antagonists (*15*, *25*). On the other hand, some evidence has been obtained for the hPR, that the C-terminus may contain a repressor function (*13*). However, no other data have been obtained which could highlight the precise function of the C-terminal sequence, and not even the crystal structure data could help to establish its precise role. These last C-terminal residues could not be resolved either in the 9-*cis*-retinoic acid receptor (RXR) or in the retinoic acid receptor (RAR) crystal structures and were said to represent a highly flexible region in the molecule (*5*, *7*). Thereafter, the first steroid LBD crystal structure for the human progesterone receptor (hPR) bound to progesterone showed the presence of the 12-residue C-terminal end tightly fixed with the loop between H8 and H9 by a short antiparallel $\beta$-sheet interaction (*9*). In the very recently described hAR LBD structure complexed with the agonist R1881 (*10*), the hAR C-terminal end is also fixed in position by a similar antiparallel $\beta$-sheet, but no further indication on the structure has been described which could explain its role.

Altogether, previous data have indicated the importance of the extreme C-terminal end in ligand binding. In this report, we present the first systematic study performed on this area of a steroid receptor, and our aim was to determine which amino acids in the C-terminal end of the hAR were most involved in the receptor function, using alanine scanning mutagenesis over a 13-residue region of hAR.

## MATERIALS AND METHODS

*Chemicals.* [$^3$H]R1881 (85 Ci/mmol) and unlabeled R1881 (methyltrienolone) were purchased from DuPont NEN (Zaventem, Belgium). RU486 (RU38486, mifepristone) was a gift from Roussel Uclaf (Romainville, France). Cyproterone acetate and hydroxyflutamide were from Schering AG (Berlin, Germany). Redivue L-[$^{35}$S]methionine (>1000 mCi/mM) and [$^{14}$C]methylated proteins were obtained from Amersham (Buckinghamshire, U.K.). Trypsin (type XIII) and ampicillin were purchased from Sigma (Saint Quentin Fallavier, France). Entensifier was from NEN. Wizard Plus SV minipreps DNA Purification System and Wizard Pure-Fection Plasmid Purification System were from Promega (Charbonnières, France). All media and chemicals for cell culture were purchased from Gibco-BRL (Life Technologies, Cergy Pontoise, France).

*Plasmids.* The pSG5-hAR was created in the laboratory by Dr. Patrick Balaguer according to the protocol described by Shemshedini et al. (*26*). The pFC31-luc was a gift of Dr. Hélène Richard-Foy (LBME/IBCG, CNRS, Toulouse, France, *27*). The $\beta$-galactosidase expression vector pCMV-$\beta$gal containing the $\beta$-galactosidase coding sequence under the control of the strong cytomegalovirus (CMV) promoter was kindly provided by Professor Pierre Chambon (IGBMC, Strasbourg, France; *28*).

*Site-Directed Mutagenesis.* Single base mutations in the hAR LBD were created by in vitro site-directed mutagenesis using the QuikChange site-directed mutagenesis kit from Stratagene according to the manufacturer's instruction with PfuTurbo DNA polymerase and complementary single-stranded pairs of mutagenic primers (Sigma-genosys Ltd., Cambridgeshire, U.K.). The C-terminal deletion was created by using the same mutagenesis kit with the following primer to remove the last 15 amino acids of the hAR: 5′-GCA GAG ATC ATC TCT GTG CAA GTG CCC TGA AGC ATT GGA AAC CCT ATT TCC-3′ and its corresponding reverse complementary primer. All constructs were checked by automated DNA sequencing. In this study, these mutants will be referred to by the wild-type residue as a single-letter code, followed by their position in the primary sequence of hAR, and followed by the replacing residue, in most cases A for alanine, except when specifically expressed.

*Binding Assays.* Cos-7 cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/mL penicillin, and 100 $\mu$g/mL streptomycin in a humidified 5% $CO_2$ incubator. Cells were seeded in 12-well tissue culture plates at a concentration of $10^5$ Cos-7 cells per well. After 6 h at

37 °C, cells were transfected with 50 ng of wild-type or mutant hAR expression vectors using the calcium phosphate coprecipitation method (*29*). After 48 h incubation at 37 °C, cells were subsequently incubated for 2 h at 37 °C with varying concentrations of [$^3$H]R1881 or [$^3$H]R1881 plus a 100-fold excess of unlabeled R1881. Cells were washed twice with cold PBS and lysed with 300 $\mu$L of 25 mM Tris−phosphate, pH 7.8, 2 mM EDTA, 10% glycerol, and 1% Triton X-100. Total [$^3$H]R1881 in a 100 $\mu$L aliquot duplicate was determined by scintillation counting. Nonspecific binding was assessed in duplicate exposed to a 100-fold excess of unlabeled R1881 as well as [$^3$H]R1881. Protein content of the cell solutions was measured by Lowry's method (*30*). Specific bound R1881 was calculated as the difference between total and nonspecific binding. Three independent assays were performed in duplicate. The Scatchard plot was obtained by plotting the amount of androgen specifically bound against the bound/free ratio. All calculations were done using Excel (Microsoft Corp., Redmond, WA).

*Transactivation Assays.* Monkey kidney CV1 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies), supplemented with 10% heat-inactivated fetal calf serum, 100 IU/mL penicillin, and 100 $\mu$g/mL streptomycin in a humidified 5% $CO_2$ incubator. CV1 cells were seeded in 12-well tissue culture plates ($2 \times 10^5$ cells per well) and transfected 8 h after using the calcium phosphate coprecipitation method (*29*) with 100 ng of hAR expression vector pSG5hAR wt or mutants, 1 $\mu$g of MMTV−luciferase reporter vector, pFC31-luc, and 250 ng of pCMV-$\beta$-galactosidase as internal control. After 16 h of transfection, cells were treated with either the synthetic nonmetabolizable androgen agonist R1881 or various antiandrogens for 30 h in serum-free DMEM. Cells were lysed by addition of 300 $\mu$L of lysis buffer (25 mM Tris−phosphate, pH 7.8, 2 mM EDTA, 10% glycerol, and 1% Triton X-100). Cells were scraped, and after one freeze−thaw cycle, the cell lysate was cleared by centrifugation at 12000*g* for 10 min. Cell lysates (100 $\mu$L) were added to 100 $\mu$L of luciferase substrate [20 mM Tricine, pH 7.8, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 1 mM EDTA, 0.53 mM ATP, 0.47 mM luciferin, and 0.27 mM coenzyme A], and luciferase activity was measured with a LKB luminometer. At least two independent assays were performed in duplicate. Transfection efficiency was assessed by $\beta$-Gal activity and luciferase activity normalized to the protein content of the cell lysates. Total protein in the supernatant was quantified (*30*) with bovine serum albumin as the standard.

*In Vitro Transcription and Translation.* In vitro transcription and translation were performed in the presence of [$^{35}$S]-methionine with the TNT T7 Quick kit from Promega (Charbonnières, France) during 1 h at 30 °C according to the manufacturer's instruction.

*Limited Trypsinization Assay.* Two microliters of labeled translation mixture was incubated at 25 °C with 1 $\mu$M ligand solution for 30 min. Limited trypsinization was performed by addition of trypsin (100 $\mu$g/mL) at 25 °C for 10 min. After incubation, 20 $\mu$L of SDS sample buffer was added. Samples were electrophoresed and fluorography was performed.

*Western Analysis.* Immunoblot analysis was used to study the effect of mutation on AR protein production. Equal volumes of 2× SDS−PAGE gel-loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) were added to the cell extracts, and the samples were heated to 100 °C for 5 min before being loaded onto a 12% SDS−PAGE gel. Electrophoresis was carried out in SDS−PAGE running buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) at 80 V for 2 h. The gel was preequilibrated for 15 min in transfer buffer (48 mM Tris−base, 39 mM glycine, 20% methanol) prior to electroblotting (100 V, 1 h) onto nitrocellulose membrane (Hybond ECL, Amersham) using the Mini Trans-Blot Cell (Biorad, Ivry-sur-Seine, France). The anti-AR antibody SpO61 diluted 1/2000 (*31*) was used to detect androgen receptor protein. Protein antibody complexes were subsequently visualized by enhanced chemiluminescence following the manufacturer's protocol (ECL system; Amersham, Les Ulis, France).

## RESULTS

*Site-Directed Mutagenesis of the C-Terminal Residues in the hAR.* We targeted different features of the hAR C-terminus using site-directed mutagenesis over a 13-residue region of the hAR (L907−Q919, Figure 1). Most residues were individually mutated into alanine, unless specified otherwise. Alanine was best suited to the scanning approach because it could accommodate most elements of the secondary structure of proteins, conveniently combining small size with minimal structural distortion. However, valine 911 was mutated into the smaller polar glycine residue instead of alanine, which was too closely related. The basic arginine residue was chosen for the mutation of the last amino acid Q919 because mutant Q919R had been described in cases of prostate cancer (*24*) and also because the last residue in all of the other steroid receptors is a basic amino acid (lysine, Figure 1). Another mutant introducing a cysteine residue at the end of the receptor—Q919C—was also produced. Residue G909 was mutated into both alanine for the alanine scanning and glutamic acid because a prostate cancer was described with this latter mutation (*22*). To check that the mutated hAR proteins were correctly expressed, they were first produced in vitro in reticulocyte lysate. Thereafter, they were expressed in living cells and transiently transfected in COS-7 cells and then quantified by Western blotting using SpO61, an hAR-specific antiserum (*31*). In this system, all mutants were produced at levels comparable with that of wild type (data not shown).

*Ligand Binding Affinities.* Binding to the mutant androgen receptors was assayed by their ability to bind the radiolabeled synthetic nonmetabolizable androgen agonist R1881 in transiently transfected Cos-7 cells. Binding affinities listed in Table 1 represent apparent equilibrium binding constants ($K_d$) determined by Scatchard analysis. Four mutant groups could be distinguished. The first group included those mutant receptors which were able to bind R1881 with the same affinity as the wt hAR ($K_d$ = 0.5 nM): K910A, K912A, Q919C, and Q919R. An intermediate group comprised the mutant receptors which were characterized by a $K_d$ between 0.5 and 1 nM: G909A, G909E, Y915A, and H917A. Another group with $K_d$s between 1 and 5 nM included I914A and V911G. The last group of mutant receptors included L907A and F916A which bound R1881 with $K_d$s higher than 5 nM (7.3 and 37 nM, respectively).

Table 1: Summary of Wild-Type and Mutant hAR Receptors, Ligand Binding Affinities, and Transcriptional Activation[a]

| receptor | binding affinity $K_d$ (nM) | MMTV-luc EC50 (nM) |
|---|---|---|
| wt | 0.47 ± 0.05 | 0.020 ± 0.009 |
| L907A | 7.37 ± 0.50 | nd |
| G909A | 1.10± 0.14 | 0.80 ± 0.3 |
| G909E | 0.80 ± 0.15 | 1 ± 0.5 |
| K910A | 0.50 ± 0.16 | 0.020 ± 0.008 |
| V911G | 3.55 ± 0.41 | 35 ± 5.5 |
| K912A | 0.50 ± 0.15 | 0.035 ± 0.007 |
| I914A | 2.40 ± 0.07 | 30 ± 5 |
| Y915A | 0.98 ± 0.14 | 0.25 ± 0.05 |
| F916A | 37 ± 5.2 | nd |
| F916P | nd | nd |
| F916Y | 0.55 ± 0.07 | 0.040 ± 0.008 |
| H917A | 1.06 ± 0.08 | 0.32 ± 0.12 |
| Q919C | 0.45 ± 0.07 | 0.035 ± 0.020 |
| Q919R | 0.47 ± 0.09 | 0.040 ± 0.050 |

[a] Apparent equilibrium binding affinities measured as $K_d$ values were determined in Cos-7 cells at 37 °C using wild-type or mutant receptors. The $K_d$ values represent a mean value ± SD obtained from three different experiments performed in duplicate. Transcriptional activity was examined by cotransfection of hAR expression plasmids and a MMTV-luc reporter plasmid and was determined in CV1 cells. The EC50 values represent a mean value ± SD obtained from two experiments performed in duplicate.

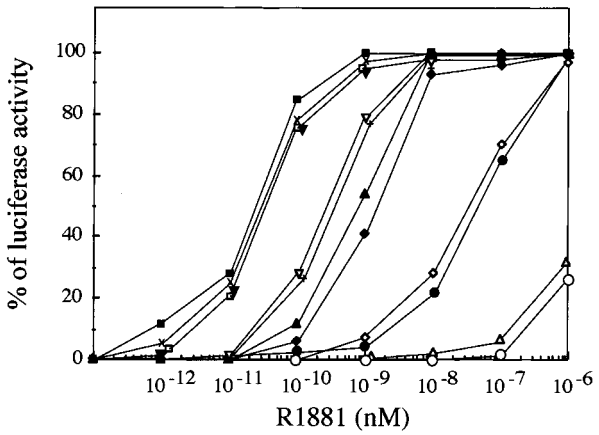


FIGURE 2: Transcriptional activity of wt hAR and mutants in response to agonist R1881. Luc activity was determined in CV1 cells cotransfected with an expression vector encoding either wt or mutated AR and a reporter plasmid, pFC31-luc, according to the method described under Materials and Methods. After transfection, the cells were then treated with increasing R1881 concentrations. Luc activity in the presence of wt hAR and $10^{-6}$ M R1881 is arbitrarily set at 100%. The results represent a typical experiment performed in duplicate. Symbols: ■, wt; ×, Q919R and Q919C; □, K910A and K912A; ▼, F916Y; ▽, Y915A; +, H917A; ▲, G909A; ◆, G909E; ◇, I914A; ●, V911G; △, L907A; ○, F916A.

The drastic drop in affinity binding of mutant F916A prompted us to investigate more thoroughly this position. For that purpose, a new mutant was produced which introduced tyrosine, another aromatic amino acid, instead of phenylalanine at position 916. During the site-directed mutagenesis, another mutant was produced which was characterized as F916P. Both mutants F916Y and F916P were expressed at levels comparable with that of wild type, and they were characterized in the same conditions as described above. F916P was unable to bind 10 nM R1881 while F916Y bound R1881 with an affinity ($K_d$ = 0.7 nM) comparable to that of wt.

The ligand binding assays demonstrated that only three positions out of the ten mutated along the C-terminal end were not affected by mutations relative to ligand binding. All of the other mutations induced a significant decrease in ligand binding, the most severe drop being triggered by the mutation of F916 into alanine, and the data clearly demonstrate that the phenyl ring on position 916 is of prime importance in the hAR for ligand binding.

*Transcriptional Activation.* Abilities of the receptor mutants to transactivate a reporter gene were determined in CV1 cells transiently cotransfected with a mouse mammary tumor virus (MMTV)−luciferase reporter gene and the corresponding mutant AR expression vector. The transfected cultures were subsequently exposed to increasing doses of the synthetic, nonmetabolizable, androgen R1881. The reporter luciferase activity was induced in a dose-dependent manner (Figure 2). Four receptor mutant groups could be distinguished according to their measured EC50 (Table 1). The first group included K910A, K912A, F916Y, Q919C, and Q919R, which had an activity either similar to or not significantly lower than that of the wt hAR, and their EC50 were at the most slightly increased as compared to wt (Table 1: 0.02−0.04 nM compared to 0.02 nM for wt). A second group was made up of four mutants—Y915A, H917A, G909A, G909E—which had an activity diminished when compared to the wt. They were able to activate the transcription only at a nanomolar concentration range (EC50 from 0.25 to 2 nM, 10−100 times the wt EC50). The two mutants V911G and I914A were part of a third group with transactivation EC50 strongly shifted toward higher concentrations (around 30 nM). The last group comprised the mutant receptors L907A and F916A, which were unable to activate the transcription at a level similar to that of wt. Their transcriptional activity remained low (about 25−30% of max) at 1 $\mu$M R1881 (Figure 2), which made the determination of their EC50 impossible.

The transactivation assays indicated that most mutants with a decreased binding affinity needed higher agonist concentrations to activate transcription (EC50s were shifted). However, both L907A and F916A mutants could not activate the transcription efficiently (transactivation max could not be reached).

*Effect of the C-Terminal End on the Conformation of the hAR.* To better understand the role of the C-terminal end on the conformation of the hAR, a conformational study by limited proteolysis was performed with the mutants characterized by a severe decreased binding affinity. Mutants L907A, I914A, and F916A were especially chosen as representative mutants for this study and produced in vitro in the reticulocyte lysate system.

When they were incubated with trypsin in the absence of ligand, hAR wt and mutants were completely degraded (data not shown). Addition of the agonist R1881 at a concentration of 1 $\mu$M before proteolysis induced a change in the conformation of the hAR wt, and two main resisting fragments could be observed as previously described (*4*, *12*): a major fragment of size 29 kDa which corresponds to the active agonist-induced conformation and a minor 35 kDa fragment which corresponds to an inactive conformation of the receptor (Figure 3A, lane 2). When mutants L907A and I914A were incubated with the same R1881 concentration (1 $\mu$M) and subsequently trypsinized, both 29 and 35 kDa

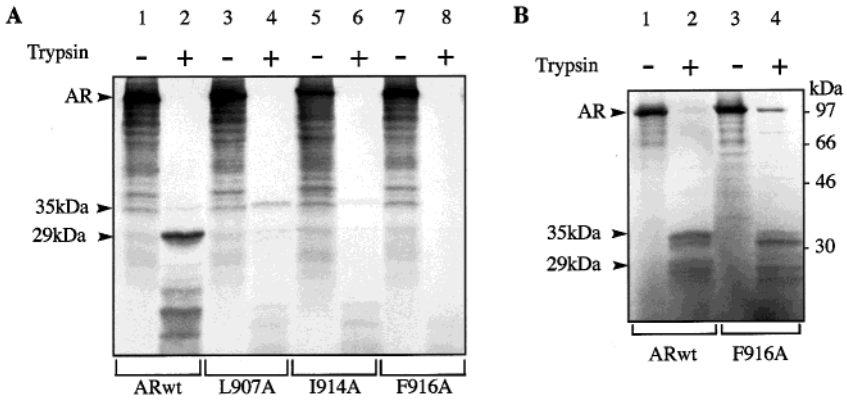


FIGURE 3: Limited trypsinization of androgen- and antiandrogen-bound wt and mutated hARs. (A) In vitro produced [$^{35}$S]hAR in the reticulocyte lysate system (wt and mutants L907A, I914A, and F916A) were incubated for 30 min at 25 °C with $10^{-6}$ M R1881 before limited tryptic (100 $\mu$g/mL) digestion for 10 min at 25 °C. The trypsin-treated samples were denatured and electrophoresed on a 12% acrylamide gel. Fluorography was subsequently performed. (B) In vitro produced [$^{35}$S]hAR (wt and mutant F916A) were incubated for 30 min at 25 °C with $10^{-6}$ M RU486 before limited tryptic (100 $\mu$g/mL) digestion for 10 min at 25 °C. The trypsin-treated samples were then treated similarly as in (A). The positions of molecular mass markers (in kDa) are shown on the right.

fragments were produced, but contrary to the wt, the 35 kDa fragment was predominantly produced (Figure 3A, lanes 4 and 6). Trypsinization of mutant F916A in the same conditions indicated that the protein was fully degraded: no resisting fragment could be observed (Figure 3A, lane 8).

Then the action of antiandrogens was tested in the same conditions of proteolysis. Both hydroxyflutamide (1 $\mu$M) and cyproterone acetate (1 $\mu$M) induced resistance of a 35 kDa fragment against trypsinization in the case of mutants L907A and I914A as is described for the wt hAR (*12*) while mutant F916A was completely degraded (data not shown). Only the presence of 1 $\mu$M RU486 before proteolysis of mutant F916A induced the formation of several resisting fragments whose sizes ranged from 35 to 20 kDa (Figure 3B, lane 4). This result demonstrates that mutant F916A is capable of binding RU486 only. This assumption is in agreement with the binding of RU486 to hAR not involving the C-terminal end of the LBD (*12*). As most of the fragments obtained with mutant F916 in the presence of RU486 were smaller than the fragments obtained in the same conditions with wt hAR (Figure 3B, lane 2), this suggests that a cleavage site located in the C-terminus has been made available in this terminal region due to the specific conformation adopted by the mutant. In fact, this cleavage site present in the C-terminus of the mutated hAR can only be observed in the presence of the RU486 molecule: actually there is no possible comparison with the proteolysis of F916A in the presence of the agonist which cannot be bound in such conditions.

The data produced by the proteolysis assays demonstrated that, at high ligand concentrations, the mutant receptors, L907A and I914A, were able to bind both agonists and antagonists, but contrary to the wt hAR, the favored conformation with these mutants was in both cases the inactive conformation of the receptor. Mutant F916A was completely degraded by proteolysis, indicating that the receptor was unable to adopt a stable conformation which could protect the protein from proteolytic cleavage, except in the presence of the androgen antagonist RU486.

*Is the C-Terminal End of hAR Involved in a Repressor Function?* To check any possible repressor role of the extreme C-terminus of the hAR, as had been previously indicated for the hPR (*13*), a C-terminal deleted mutant—ΔC15 hAR—was produced by deleting the last 15 amino acids of the hAR. It was previously demonstrated that the hAR mutant deleted of the last 12 amino acids was able to bind RU486, but this mutant did not activate transcription (*12*). However, some essential residues for the repressor function of hPR are located upstream of the ΔC12 cleavage (see Figure 1).

The ΔC15 hAR mutant was expressed at a level comparable with wild-type, and it was characterized in the same conditions described above for the site-directed mutants. The ΔC15 hAR was unable to bind the agonist R1881, and it was devoid of any activity in the presence of R1881. These results are in perfect agreement with those previously demonstrated with the hAR mutant deleted of the last 12 amino acids (*12*). When transactivation assays were then performed in the presence of the antagonist RU486, no significant increased activity was observed with the ΔC15 hAR as compared to the wt hAR. In our study, 1 $\mu$M RU486 showed a limited partial agonistic effect with wt hAR (15% of the R1881 activity, as described in ref *12*), and the RU486 partial agonistic effect observed with ΔC15 mutant was about 10%.

These results indicate that even though RU486 can be bound by a C-terminal deleted hAR, this antagonist still remains inactive toward receptor activation. Similar results had been obtained with carboxyl-end truncated rGR mutants which were not activated by RU486 (*32*). We then assume that if a repressor function exists in hAR, it is not located in the 15 last residues constituting the receptor C-terminal end.

## DISCUSSION

Although the extreme C-terminal end of the LBD has never been described as being fully part of the ligand binding pocket, our results clearly demonstrate its direct involvement in the ligand binding activity of the hAR. Actually, most of the point mutations decribed in this paper did indeed affect the binding, however, at different levels depending on the position. As a consequence, the transcriptional activity of the various hAR mutants was modified accordingly. Only three positions among the residues tested in our study were not affected by point mutations: K910, K912, and the last residue, Q919. Furthermore, our results demonstrate how specific the location of each amino acid is in this region of

the receptor, and they also highlight essential criteria needed for the function of this end part of the receptor.

The first criterion is related to the precise position of the extreme C-terminus. A previous report had already pointed to the presence of a conserved proline, P780 in rGR (equivalent position P904 in hAR), for providing the correct orientation of the carboxyl tail (*32*). Our results indicate a similar role for glycine at position G909. In fact, due to the unique conformational flexibility of the small glycine residue, any mutation of this position modifies the receptor's properties (alanine, this study; glutamic acid, this study and ref *22*; arginine, ref *19*), indicating that G909 is important for the correct active orientation of the C-terminus. As for the other proline residue located at position 913, it is shown in the crystal structure (*10*) to be involved in a $\beta$-sheet interaction with loop H8−H9, which certainly triggers the orientation of essential residues.

The second criterion for providing hAR with a correct ligand binding conformation is hydrophobicity, namely, at positions L907, V911, and I914. The mutation of leucine 907 by alanine had a really drastic effect on ligand binding while the previously described substitution of leucine into phenylalanine (CAIS, *18*) was not so drastic, an effect related to both the hydrophobicity and steric volume of the side chain groups. Moreover, although not conserved, the corresponding positions to V911 and I914 in other steroid receptors are all occupied by hydrophobic residues. The substitution of I914 by the smaller alanine and V911 by the even smaller glycine provoked a modification of their binding affinity toward R1881. The binding properties of all of these mutants were decreased because the active ligand binding conformation is not favored, thus indicating the necessity of hydrophobic interactions in this area of the receptor to provide the active conformation.

The most critical feature is the presence of the aromatic phenylalanine in position 916. Upon proteolysis, mutant F916A was degraded, indicating that the proper compaction of the LBD could not occur after agonist binding. The specific case of RU486, the only ligand able to protect mutant F916A from trypsin degradation, provides additional evidence for a different binding mechanism of RU486, supporting the fact that the C-terminus of the receptor is not required for its binding (*12*). These results demonstrate the involvement of F916 in the process of providing the active conformation of the receptor. Moreover, our study demonstrates that the location of the aromatic ring is strictly specific to position 916 because no such dramatic change in receptor function could be observed when Y915 was substituted. Additionnally, substitution of F916 by a tyrosine produced a mutant with no significant changes when compared to the wt. This phenylalanine, the fourth amino acid from the end, is a conserved residue among the other steroid receptors. A previous report had demonstrated that the deletion of the last four residues of the hMR abrogated all binding (*15*) and that the mutation F981A in the hMR provoked a 20-fold decrease in the affinity for aldosterone. Another study with the analogous phenylalanine in the mGR—F780—mutated into alanine also indicated that the phenyl group is an important determinant of ligand binding affinity (*25*). Because of an accessible center of negative charge (from the ring $\pi$ electrons), aromatic rings are able to participate not only in the obvious, hydrophobic bonding and van der Waals interactions but also in amino−aromatic bonding or aromatic stacking interaction (*33*, *34*). By examination of the homology model of hAR based on the hPR crystal structure (*35*), two residues are in close proximity to F916: R831 located in helix 9 and D864 located in helix 10. As F916, these two residues are conserved throughout the steroid receptor family including AR, GR, MR, and PR. Furthermore, like F916, a number of mutations of either R831 or D864 are associated to CAIS (*16*, *17*), thus demonstrating their importance for the ligand binding function of the receptor. Due to their close proximity, an amino−aromatic interaction could occur between F916 and R831, but a similar hypothesis is not possible between F916 and D864. However, the area surrounding D864 is a hydrophobic patch on helix 10 which could allow hydrophobic interactions with the C-end and H10. Such interactions could contribute to the positioning of both helix 10 and helix 12 which appear to stabilize androgen in the binding pocket upon ligand binding.

Due to the strong homology between steroid receptors in the C-terminal end, we assume that the criteria found in our study, such as flexibility at strategic positions, hydrophobicity on homologous positions to V911 and I914, apply to other steroid receptors, as is the case for the aromaticity of position F916. Our data most especially indicate that the C-terminal end makes important contributions to the stability of the hAR LBD tertiary structure by the means of hydrophobic patches which are not included in the ligand binding pocket.

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