A new mutation of the androgen receptor, P817A, causing partial androgen insensitivity syndrome: *in vitro* and structural analysis

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Abstract

Androgen insensitivity syndrome (AIS) is an X-linked disease caused by mutations in the androgen receptor (AR) resulting in various degrees of defective masculinization in 46,XY individuals. In the present study, we describe a novel mutation in exon 7 of the AR gene in an Egyptian patient with partial AIS (PAIS). Sequencing analysis of the AR gene revealed a novel missense mutation, P817A, within the ligand-binding domain (LBD). This is the first report of a mutation within the short amino acid motif (codons 815–817) of the β -strand lying between helices H8 and H9 of the AR LBD. The functional defects of the mutated protein were characterized by *in vitro* study and included significantly decreased ligand-binding affinity and impaired transactivation potential. Limited proteolysis assays performed with the wild-type and mutant AR receptors incubated with the synthetic agonist R1881 revealed that the P817A mutation resulted in a reduced stabilization of the AR active conformation. Structural analyses showed that this mutation is likely to perturb the β -sheet interaction between residues 815–817 and 911–913. This structural alteration destabilizes the position of the C-terminal extension, which contains residues critical for androgen function.

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Introduction

The androgen receptor (AR) belongs to the steroid/nuclear receptor superfamily of ligand-dependent transcription factors (Evans 1988). It mediates the biologic effects of the endogenous androgens, testosterone (T) and dihydrotestosterone (DHT), and regulates the genes for male differentiation and development. The AR is encoded by the AR gene, which is located on Xq11–12 and consists of eight exons (Trapman *et al.* 1988, Lubahn *et al.* 1989). Exon 1 codes for a large amino-terminal domain; this is the least conserved region of the steroid receptors and is

involved in transcriptional activation of target genes. The DNA-binding domain is encoded by exons 2 and 3 and contains two zinc-finger motifs (Freedman 1992). The carboxyl terminal encodes for the ligand-binding domain (LBD) and also encompasses subdomains involved in dimerization and transcriptional activation (Simental *et al.* 1992, Wong *et al.* 1993, Jenster *et al.* 1997).

About 300 different AR mutations have been reported (the AR gene mutation database: www.mcgill.ca/androgendb/) in androgen insensitivity syndrome (AIS), one of the most common causes of undermasculinization in 46,XY individuals. The phenotypic spectrum of AIS ranges from

normal female phenotype in complete AIS (CAIS), through female phenotype with clitoromegaly, to male phenotype with ambiguous genitalia in partial AIS (PAIS) or isolated infertility in individuals with normal male external genitalia (minimal AIS) (Quigley *et al.* 1995). The vast majority of AR abnormalities in AIS are missense mutations. These single-residue defects provide the basis for detailed study of the AR structure–function relationship. The structural approach, in complement to the classic *in vitro* studies of AR functional capabilities, is a powerful tool for structure–function investigations (Shkolny *et al.* 1999, Poujol *et al.* 2000, Rosa *et al.* 2002).

The crystal structures of the unliganded (apo) and liganded (holo) LBDs of various nuclear receptors, including the AR, have been solved (Bourguet *et al.* 2000). They revealed a similar fold in the 12 α -helices with the major difference between the apo- and holo-state being the position of the helix H12 that encompasses residues of the domain of the transcription activation function 2 (AF-2). The typical conformation of an agonist-bound nuclear receptor reveals that helix H12 is precisely positioned as a ‘lid’ for the LBD (Wurtz *et al.* 1996, Matias *et al.* 2002). Conversely, to decrease the structural constraints of the ligand on the receptor, the conformation of the LBD has to accommodate the ligand by a change in the orientation of helix H12 (Brzozowski *et al.* 1997, Shiau *et al.* 1998). In a recent study (Tahiri *et al.* 2001), we demonstrated that the last 13 amino acids located after helix H12 are important in ligand binding and AR activation because they stabilize the active conformation of the receptor, even though they are not part of the ligand-binding pocket (LBP). Here we report the identification of a new missense mutation (P817A) within the AR LBD in a patient with PAIS. *In vitro* experiments confirmed that the substitution of proline at position 817 altered AR function. Structural analysis suggested that the altered functionality of the P817A mutant is due to the destabilization of the C-terminal extension.

Materials and methods

Patient

The patient was referred at the age of 7 months for ambiguous genitalia, although he was being raised

as a male. The parents were not related, and there was no family history of similar cases. Genital examination revealed the presence of two palpable testes within the labioscrotal folds, microphallus and penoscrotal hypospadias. Chromosomal analysis showed that the patient had a 46,XY karyotype. The human chorionic gonadotropin (hCG) stimulation test was performed to assess testicular T biosynthesis and conversion to DHT. The basal T value of 0.4 ng/ml increased normally to 4.9 ng/ml, and the T/DHT ratio was normal at 8.9.

Identification of the mutation

DNA was extracted from peripheral blood leukocytes. Exons 2 to 8 of the AR were amplified by polymerase chain reaction (PCR) using sets of primers and reactions previously described (Lumbroso *et al.* 1996). PCRs were verified for correct length on agarose gel, purified with Qiaquick PCR columns (Qiagen, Courtaboeuf, France) and sequenced with the ABI Prism Big Dye terminator sequencing kit and the ABI 310 genetic analyzer (Applied Biosystems, Courtaboeuf, France).

Plasmid construction

The hAR mutation P817A was introduced into the wild-type (WT) hAR expression vector pSG5-hAR by PCR-based point mutagenesis, using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions. The primer pair used was A1 5′-GCT ACT CTT CAG CATT ATT GCA GTG GAT GGG CTG-3′ and A2 (the corresponding reverse complementary primer). pFC31 Luc, which contains the murine mammary tumor virus (MMTV) promoter driving the luciferase gene (Gouilleux *et al.* 1991), was obtained from H. Richard-Foy (LBME, Toulouse, France). For transient transfection, all plasmids were purified with nucleobond-AX cartridges (Macherey-Nagel, Hoerd, France).

Androgen-binding assays

COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen,

Cergy Pontoise, France), in a humidified atmosphere containing 5% CO₂. The apparent binding affinity of the WT hAR and mutant hAR was determined by plating 1.5×10^5 cells on 12-well tissue culture plates. After 8 h, the cells were transiently transfected with 50 ng of pSG5-WT hAR or pSG5-hAR-P817A and 200 ng of pCMV- β -galactosidase by the calcium phosphate coprecipitation method. After overnight incubation, precipitates were removed and replaced by fresh DMEM medium with 2% dextran-coated charcoal FCS (DCC-FCS) prepared in-house (Bignon *et al.* 1988). After 48 h, transfected cells were incubated for 2 h at 37 °C with increasing concentrations of [³H]R1881 from 0.02 to 4 nM for total binding. Nonspecific binding was measured in parallel incubations containing an additional 1000-fold molar excess of radioinert ligand. Aliquots of total and free labeled steroid were taken after the binding reaction. The plates were put on ice for 15 min, and labeling medium was removed. The cells were washed three times with 1 ml of cold PBS, and harvested in 400 μ l of lysis buffer (25 mM Tris phosphate, pH 7.8, 2 mM CDTA, 10% glycerol and 1% triton X-100). Specific radioactivities were determined on 100 μ l of lysate. Specific binding was determined as the difference between total and nonspecific counts. The apparent equilibrium dissociation constants (K_d) and the androgen-binding sites (B_{max}) were derived from Scatchard plots.

Immunoblot analysis

Immunoblotting was performed in the COS-7 cell line as previously described (Lobaccaro *et al.* 1999). Briefly, 2×10^6 cells were plated on 10 cm dishes and transfected with 8 μ g plasmid. Sixteen hours after transfection, the cells received fresh medium with 10^{-9} M R1881 or vehicle and were cultured for an additional 30 h before harvesting. The cells were washed three times with PBS and lysed in the presence of antiprotease (Sigma P8340) and phenyl methyl sulfonyl fluoride (PMSF). Cell lysates were subjected to electrophoresis, and gels were blotted onto Hybond membrane (Amersham Biosciences, Paris, France). hAR immunodetection was performed with the rabbit polyclonal antibody N-20 directed against a peptide corresponding to amino acids 2 to 21 mapping at the N terminus of the hAR (Santa Cruz Biotechnology, Tebu, France).

For detection of β -actin, membranes were incubated with the polyclonal antibody developed in rabbit, using the C-terminal actin fragment (Sigma). Secondary antibody (goat anti-rabbit immunoglobulin (Ig) G conjugated to horseradish peroxidase (Amersham)) was used for detection by enhanced chemiluminescence (Pierce, Interchim, Montluçon, France).

Limited proteolytic digestion of the wild-type and mutant P817A

Expression plasmids (1 μ g) (WT hAR or P817A mutant) were transcribed and translated with the TNTT7 quick-coupled transcription/translation system (Promega, Charbonnières, France), as described previously (Georget *et al.* 2002) in the presence of [³⁵S]methionine (1000 Ci/mmol; ICN, Orsay, France) for 2 h at 30 °C. A volume of 5 μ l ³⁵S-labeled receptor synthesized *in vitro* was preincubated for 30 min at 37 °C with various concentrations of R1881 (10^{-9} to 10^{-6} M). Limited proteolysis was performed by the addition of 5 μ l of various concentrations of trypsin (Sigma) (final concentrations, 25 and 50 μ g/ml). Incubations with protease were conducted for 10 min at 27 °C and stopped by addition of 10 μ l SDS sample buffer and cooling in ice. Samples were boiled for 5 min. The products of proteolysis were separated on a 12% SDS–polyacrylamide gel (SDS–PAGE). After electrophoresis, the gels were washed in distilled water and vacuum-dried for 20 min. Gels were exposed to a Fujix film imaging plate for 45 min and to autoradiography overnight. The intensity of the bands was semiquantified with Fujix software.

Transfection and luciferase activity assay

CV-1 cells were cultured in the same medium as COS-7 cells. The cells were seeded on 24-well plates (10^5 cells per well) and transfected for 8 h after using calcium phosphate with 25 ng WT hAR or mutant hAR, 125 ng pCMV- β -galactosidase and 500 ng pFC31-Luc. The precipitate was removed after 16 h, and the cells were maintained in fresh DMEM with 3% DCC-FCS, with vehicle alone or various ligand concentrations. After 30 h, the cells were lysed in 200 μ l lysis buffer described above. The luciferase activity was measured by the reaction of lysate with the luciferin solution:

Figure 1 Expression levels of P817A and WT hAR forms. COS-7 cells were transfected with expression vectors and whole extracts were analyzed by western blotting using polyclonal antibodies against hAR and β -actin as a control for equal protein loading. In the absence of ligand, expression level was similar for WT and mutant hAR. In the presence of 10^{-9} M R1881, a more intense band was observed for WT, whereas in the absence or presence of R1881, the bands for the mutant were of similar intensity.

270 μ M coenzyme A, 470 μ M luciferin, 530 μ M ATP, 20 mM tricine, pH 7.8, 1.07 mM $(\text{MgCO}_3)_4$, $\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$, 2.67 mM MgSO_4 , and 1 mM EDTA. Luciferase activity was measured on

Figure 2 Apparent equilibrium binding affinities of WT hAR and P817A mutant for $[^3\text{H}]\text{R1881}$ measured at 37°C for 2 h in transiently transfected COS-7 cells. The apparent equilibrium dissociation constants (K_d) and the number of sites (B_{max}) were derived from Scatchard plots. The results represent the mean of at least two independent determinations performed in triplicate. B_{max} was similar for WT and mutant (220 and 245 fmol/m protein respectively), whereas binding affinity for mutant was clearly decreased (2.21 vs 0.27 nM).

Figure 3 Transcriptional activity of the WT and mutant hARs. CV1 cells were cotransfected with vectors expressing receptors, an androgen-regulated gene (MMTV-Luc) and plasmid for β -galactosidase constitutive expression. The cells were incubated in the presence of increasing concentrations of R1881. Shown is the fold induction relative to activity determined in the absence of R1881. Reported values are the means ($\pm$ S.E.) of at least three independent experiments performed in duplicate. The P817A mutation ($\text{EC}_{50}=2 \cdot 10^{-9}$ M) significantly altered the transcriptional activity as compared with WT hAR ($\text{EC}_{50}=4 \cdot 10^{-11}$ M).

an Amersham Biosciences luminometer. The induction of luciferase activity is indicated in arbitrary units corrected by β -galactosidase activity, and the data are expressed as fold induction compared with vehicle. At least three independent assays were done in duplicate.

Results and Discussion

Molecular diagnosis and *in vitro* investigations

In this patient presenting ambiguous genitalia and normal testosterone production, molecular analysis confirmed the diagnosis of partial androgen insensitivity by identifying a proline to alanine substitution at position 817 within the hAR LBD. Moreover, we found that the mother was a heterozygous carrier for the mutation, so genetic counseling could be planned for future pregnancies. Carrier status will also be established if requested by female siblings in the family. To our knowledge, this mutation has never been reported.

We next performed *in vitro* experiments to assess whether the P817A mutation was indeed deleterious to hAR function and to identify which step of the hAR mechanism of action was defective.

Western-blot analysis of transfected COS-7 cells revealed normal expression for both WT and mutant hAR in the absence of ligand (Fig. 1). In the

Figure 4 Limited proteolysis experiments. *In vitro* experiments were performed with 1 µg WT hAR or 1 µg P817A mutant expression vectors. Incubation of *in vitro* produced receptors with R1881 (10^{-9} to 10^{-6} M) was followed by treatment with trypsin (25 and 50 µg/ml) for 10 min at 27 °C. Trypsin-digested products were run on a 12% polyacrylamide gel, and bands were detected by autoradiography. The autoradiogram shown is representative of three separate experiments. The intensity of the bands was semiquantified by Fujix software. The relative band intensities, which are represented in the lower panel, were calculated as the ratio between the protected band (29 kDa) and the nondigested receptor (110 kDa) incubated in each hormonal treatment.

presence of R1881, a more intense band was observed for WT hAR, whereas in the absence or presence of R1881, the bands were of similar intensity for the mutant. This indicates that the mutant hAR was less protected by R1881 and suggests a binding defect. The specific binding sites and the apparent equilibrium dissociation constants (K_d) of the receptor ligand were determined by Scatchard analyses (Fig. 2). Whereas the number of sites was similar for WT hAR and the P817A mutant ($B_{max}=220$ and 245 fmol/mg protein respectively), the P817A mutant displayed a lower affinity for R1881 ($K_d=2.21 \pm 0.5$ nM) than did WT hAR ($K_d=0.27 \pm 0.05$ nM) (Fig. 2).

To quantify the functional consequences of the mutation, transcriptional activity was measured in CV-1 cells transfected with an hAR expression vector, pSG5 hAR, and a MMTV-Luc reporter gene. Activity was measured over a range of ligand concentrations. Data are represented as fold induction of luciferase activity determined relative to the activity in absence of R1881 (Fig. 3). The P817A mutation ($EC_{50}=2 \cdot 10^{-9}$ M) significantly altered the transcriptional activity compared with WT hAR ($EC_{50}=4 \cdot 10^{-11}$ M).

The *in vitro* experiments confirmed that the mutant had impaired transactivation capacity by decreasing affinity for the ligand and was thus responsible for the defective masculinization in the patient.

Structural analysis

Previous crystallographic studies demonstrated that nuclear receptors interact with coactivators via a hydrophobic groove formed by residues from helices H3, H4 and H12 (Bledsoe *et al.* 2002, Shiau *et al.* 2002). This recognition surface is formed in the presence of agonist ligands that stabilize the active position of helix H12 over the ligand-binding cavity. This alignment of the helix H12 can be impeded or completely prevented by partial or full antagonists (Bourguet *et al.* 2000), as well as by naturally occurring mutations (Kallenberger *et al.* 2003). Limited proteolysis can evaluate the impact of a given ligand or mutation on the conformational status of the receptor in a simple and rapid experiment. It has been shown that when incubated with the agonist R1881, WT hAR is stabilized in an active conformation, providing a

Figure 5 Overall structure of the DHT-AR LBD complex. Helices are represented as blue tubes, and β -strands are depicted as arrows and colored light green. Structural elements are labeled (helices H1 to H12 and strands s1 to s4). Connecting loops are colored purple. DHT is shown in stick representation with the atoms colored by atom type (carbon, yellow; oxygen, red). The Figure depicts how the AF-2 helix (H12) folds over the ligand-binding cavity with the C-terminal extension docked between helices H9 and H10.

29 kDa resistant band (Kuil *et al.* 1995, Georget *et al.* 2002, Terouanne *et al.* 2003). We therefore investigated whether the modifications in the ligand-binding and transactivation activities observed for P817A hAR were the result of an inappropriate receptor conformation. Limited proteolysis assays were performed with the WT hAR and the mutant receptor incubated with various concentrations (10^{-9} to 10^{-6} M) of the synthetic agonist R1881 (Fig. 4). We observed that, in the presence of 10^{-9} M R1881, both WT and mutant

were completely digested. At 10^{-8} and 10^{-7} M, the P817A mutant displayed a higher sensitivity to trypsin than did WT. It should be noted that at these low R1881 concentrations, the decreased affinity of the mutant for the ligand increased the susceptibility to proteolytic digestion. However, the mutant's higher sensitivity to trypsin was also observed for saturating conditions (10^{-6} M R1881). This demonstrated that R1881 induced more stable active conformation for WT hAR than for the P817A mutant.

Figure 6 Close-up view of the specific network of interactions between the C-terminal extension (S4) and the other parts of the AR LBD (S3, H9 and H10). Residues included are either hydrogen-bonded or make van der Waals contacts, as described in the text (dotted purple lines). Side-chain atoms are colored by atom type (carbon, yellow; oxygen, red; nitrogen, blue). Residues that belong to S4 are labeled in green. P817 from S3 is labeled in red. The orange sphere represents a water molecule.

The LBDs of 3-keto-steroid receptors contain some features that differ from those of other nuclear receptors. Among them is the important role that the C-terminal extension plays in hormone binding and receptor activation in AR, progesterone receptor (PR), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (Jenster *et al.* 1991, Kuil *et al.* 1995, Xu *et al.* 1996, Zhang *et al.* 1996, Couette *et al.* 1998, Tahiri *et al.* 2001). The crystal structures of AR, PR, and GR (Williams & Sigler 1998, Matias *et al.* 2000, Sack *et al.* 2001, Bledsoe *et al.* 2002) show that these residues fold as a β -strand (S4) in the active conformation and form a conserved antiparallel β -sheet with the β -strand S3 between H8 and H9 (Fig. 5). In AR, this 13-residue peptide is tightly held in place not only by the β -sheet interaction between residues 911–913 (S4) and 815–817 (S3),

which involves hydrogen bonds between main chain NH and CO atoms of both adjacent strands (Fig. 6), but also by the interaction of some of the residue side-chains with the LBD core. In particular, V911, I914 and F916 are oriented such that they are engaged in strong van der Waals contacts with residue side-chains from helices H9, H10 and H5 (Fig. 6). In addition, D864 (H10) and R831 (H9, *via* a water molecule) are hydrogen-bonded to a main-chain peptide bond of the C-terminus of AR. Interestingly, most of the residues involved in the stabilizing interactions described above are specifically conserved in the subfamily of 3-keto-steroid receptors. Moreover, mutational analysis of hAR LBD showed that, of the 13 residues mutated in the C-terminal extension, the replacement of F916, I914 and V911 induced the most drastic drop in the ligand-binding

affinity and receptor activation, thus suggesting the physiologic relevance of the observed structures (Tahiri *et al.* 2001). The impact of the P817A mutation on hAR activity highlights this particular feature of the 3-keto-steroid receptors. Taken together, our results support the notion that mutating the proline 817 in S3 of hAR to alanine significantly decreases the stability of the active conformation. This could alter the recruitment of coactivators and hence the ability of hAR to transactivate. The observation that P817 is conserved in all steroid receptors, except the estrogen receptor, suggests a specific structural role for this proline residue in the 3-keto-receptor subfamily. The presence of a proline residue at this particular position is probably important to maintain the β -strand conformation of S3. In the P817A mutant, the specific rotational constraints conferred by a proline residue are relieved, leading to the disruption of both the β -strand conformation of S3 and the stabilizing β -sheet interaction with S4. This confirms that the particular position and conformation adopted by the C-terminal extension in the crystallographic structures are required for 3-keto-receptor activation. In conclusion, the naturally occurring mutation at P817 reveals a new example of a residue that is not in contact with the ligand and does not belong to AF-2, but which does have an important role in ligand binding and receptor activation by stabilizing the helix H12 in the active conformation.

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