Human androgen receptor gene ligand-binding-domain mutations leading to disrupted interaction between the N- and C-terminal domains

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Abstract

Most mutations in the androgen receptor (AR) ligand-binding domain (LBD) disrupt binding of the natural ligands: dihydrotestosterone and testosterone. Some AR LBD mutations do not affect ligand binding but they disrupt androgen-induced interaction of the N-terminal motif FXXLF and C-terminal activation function 2 (AF2). As N-/C-terminal interaction requires binding of agonists that have androgen activity *in vivo*, it correlates well with the phenotype. To study this further, we searched the Cambridge intersex database for patients with a detected missense mutation in the AR LBD presenting with normal ligand binding. Six mutations (D695N, Y763C, R774H, Q798E, R855H and L907F) were selected and introduced by site-directed mutagenesis into the pSVAR and pM-LBD plasmids. The transactivational potential of the wild-type and mutant androgen receptors (pSVAR) was examined by dual-luciferase assay using pGRE-LUC as a reporter vector. N-/C-terminal interaction was studied by mammalian two-hybrid assay using wild-type and mutated AR LBD (pM-LBD), pVP16-rAR-(5–538) (encoding rat amino-terminal AR) and pCMX-UAS-TK-LUC as a reporter. AR LBD mutations D695N, R774H and L907F presented with minimal transactivational capacity and N-/C-terminal interaction was totally disrupted. Mutations Y763C and R885H had some residual dose-dependent transactivational potential and minimal N-/C-terminal interaction. Q798E presented with good transactivational potential and it showed only mild reduction in N-/C-terminal interaction. With the selected mutations, N-/C-terminal interaction correlated well with AR transactivation and the phenotype. Disrupted N-/C-terminal interaction is capable of providing the mechanism for androgen-insensitivity syndrome in most cases where the mutation in the LBD does not disrupt ligand binding. Furthermore, mutations leading to the disrupted N-/C-terminal interaction can be localized to certain critical regions in the three-dimensional structure of the AR LBD. Our study shows that apart from the previously reported regions, regions just before helix 3, between helices 5 and 6, and at helix 10 are also important for AR N-/C-terminal interaction.

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Introduction

The human androgen receptor (hAR) is a member of the steroid hormone subfamily of nuclear transcription factors (Mangelsdorf *et al.* 1995). It mediates male sexual differentiation and the development and maintenance of male (and some female) sexual characteristics. More than 300 mutations in the X-linked AR gene result in androgen-insensitivity syndrome (AIS), comprising a clinical continuum of virilization disorders ranging from mild AIS to complete AIS (CAIS; Gottlieb *et al.* 2004).

Together with other steroid receptors, the AR has a well-characterized modular structure. The protein is comprised of a polymorphic N-terminal domain (NTD), a central well-conserved DNA-binding domain and a

C-terminal ligand-binding domain (LBD; Ikonen *et al.* 1997, Tsai & O'Malley 1994). The AR LBD comprises 12 helices, and the ligand-binding pocket is formed by helices 3, 4, 5, 7, 11 and 12 together with the β -sheet preceding helix 6 (Matias *et al.* 2000). The hAR NTD harbors hormone-independent transcription activation function 1 (AF1; Ikonen *et al.* 1997). Conserved FXXLF (FQNLF; residues 23–27) and WXXLF (WHTLF; residues 433–437) motifs are also located within the NTD, forming in part the interface for the interaction of the NTD with hormone-dependent AF2 in the LBD (He *et al.* 2000). Both of these motifs are required for the N-/C-terminal interaction, but FXXLF is predominant for the interaction. The N-/C-terminal interaction maintains AR activation at low circulating androgen concentrations by holding the ligand in the ligand-

Table 1 Cambridge intersex database patients with a detected missense mutation in the AR LBD and normal androgen-binding capacity on genital skin fibroblasts. Local reference values are also given. For details, see Batch *et al.* 1992.

	Phenotype	External genitalia	$B_{\max}$ ($\times 10^{-18}$ mol/ μ g DNA)	K_d ($\times 10^{-10}$ mol/l)
AR genotype				
WT	Normal male	Normal masculine	200–589	0.95–2.03
D695N	CAIS	Normal feminine	511	0.3
Y763C	PAIS (male)	Bifid scrotum, perineal hypospadia, micropenis	1992	3.0
R774H	CAIS	Normal feminine	270	1.4
Q798E	PAIS (female)	Bifid scrotum, perineal hypospadia, micropenis	1756	1.4
R855H	CAIS	Normal feminine	351	0.9
L907F	CAIS	Normal feminine	264	1.4

$B_{\max}$, maximal ligand-binding capacity; K_d , ligand-binding affinity.

binding pocket (He *et al.* 1999, Ikonen *et al.* 1997). The AF2 of the AR is weak due to its reduced ability to recruit p160 coactivators such as steroid receptor coactivator 1 (SRC1), transcriptional intermediary factor 2 (TIF2, SCR2) and the SCR3/TRAM1/AIB1/pCIP/ACTR/RAC3 group of coactivators. AR N-/C-terminal interaction further competitively reduces this recruitment but overexpression of p160 coactivators may shift this equilibrium to overcome N-/C-terminal interaction-mediated inhibition of p160 coactivator recruitment (He *et al.* 2001).

There is evidence indicating that disrupted interaction between the hAR N- and C-termini can serve as a mechanism for AIS also in cases where ligand binding is normal (Elhaji *et al.* 2004, Ghali *et al.* 2003, Langley *et al.* 1998, Quigley *et al.* 2004, Thompson *et al.* 2001). The AR N-/C-terminal interaction is selectively induced by ligands that have AR agonist activity *in vivo* including the biologically active androgens 5 α -dihydrotestosterone (DHT) and testosterone (Zhou *et al.* 1995). It is not induced by ligands that bind the AR and induce its nuclear transport but who fail to activate the AR target genes.

The mutations critical for N-/C-terminal interaction have been clustered in certain regions of the hAR LBD crystal structure (Matias *et al.* 2000). The LBD has a hydrophobic groove formed by helices 3, 4, 5 and 12 and this groove serves as a binding site for the p160 coactivators and for the AR NTD. Mutations disrupting N-/C-terminal interaction have also been localized at helix 6 and between helices 10 and 11 (Ghali *et al.* 2003).

To find further hAR mutations and regions of the hAR LBD critical for this interaction, we studied whether defects in N-/C-terminal interactions can offer an explanation for the phenotype in cases with normal or near-normal androgen binding. We also studied how N-/C-terminal interaction correlates with the phenotype.

Material and methods

Patients

The Cambridge intersex database was searched for patients with a mutation in the AR LBD but having normal androgen-binding capacity. Five such patients were found, and one case with significant residual binding but subnormal ligand-binding affinity (evidenced by high K_d value) was also included (Table 1). Four patients presented with CAIS, two patients had partial AIS (PAIS), one of these two was raised female another male. Local ethics committee approval and informed consent from all the individuals in this study were obtained for the use of patient samples.

Molecular analysis

Genomic DNA was extracted from peripheral blood or genital skin fibroblasts. AR protein coding regions and flanking intronic sequences of exons 2–8 and non-polymorphic regions of exon 1 were screened by single-strand conformation polymorphism analysis and/or direct sequencing as previously described (Batch *et al.* 1992).

Binding assay

The binding assay on patients’ genital skin fibroblasts was performed as reported (Batch *et al.* 1992). The results of the assays are given in Table 1.

Plasmid constructions

The wild-type (WT) AR-encoding plasmid pSVAR has been reported previously (Brinkmann *et al.* 1989). The six LBD mutations were introduced into the pSVAR using site-directed mutagenesis PCR, and Kpn1/PsiI

Table 2 Primers and restriction enzymes (RE) used in plasmid reconstruction by *in vitro* mutagenesis. Primers 1–12 were used to incorporate the mutation into the WT AR plasmid (pSVAR) and the mutated nucleotides are indicated with bold letters. Primers 13–16 were used to amplify the first-stage fragments together with the primers carrying the mutations. Kpn1/Psi1 sense and antisense or EcoR1 sense and antisense primers were used in the second-stage (fuse) PCR as well as for sequencing the whole PCR-amplified fragment

Primer	Primer seequence	RE
D695N sense	5'-CGACAACAACCAGCCCA ACT CCTTTGCAGCC-3'	Kpn1/Psi1
D695N antisense	5'-GGCTGCAAAGGAGTTGGGCTGGTTGTTGTCG-3'	Kpn1/Psi1
Y763C sense	5'-CTCCAGGATGCTCT G CTTCGCCCTGATC-3'	Kpn1/Psi1
Y763C antisense	5'-GATCAGGGGCGAAG C AGAGCATCCTGGAG-3'	Kpn1/Psi1
R774H sense	5'-CAATGAGTACC AC ATGCACAAGTCCCGGATG-3'	Kpn1/Psi1
R774H antisense	5'-CATCCGGGACTTGTGCATGTGGTACTCATTG-3'	Kpn1/Psi1
Q798E sense	5'-GTTTGGATGGCTC GAA ATCACCCCCAGG-3'	Kpn1/Psi1
Q798E antisense	5'-CCTGGGGGGTGATTT CG AGCCATCCAAAC-3'	Kpn1/Psi1
R855H sense	5'-CATCCTGCTCAAGAC ACT TCTACCAGCTCAC-3'	EcoR1
R855H antisense	5'-GTGAGCTGGTAGAAGTGTCTTGAGCAGGATG-3'	EcoR1
L907F sense	5'-GTGCCCAAGATCTTTTCTGGGAAAGTCAAGCCC-3'	EcoR1
L907F antisense	5'-GGGCTTGACTTTCCAGAAA A GATCTTGGGCAC-3'	EcoR1
Kpn1/Psi1 sense	5'-GCGGCGAGGCGGGAGCTGTAGC-3'	Kpn1/Psi1
Kpn1/Psi1 antisense	5'-CCACCTTCTGATAGGCAG-3'	Kpn1/Psi1
EcoR1 sense	5'-GGATGCTCTACTTCGCCCTGATC-3'	EcoR1
EcoR1 antisense	5'-CCACCTTCTGATAGGCAG-3'	EcoR1

and EcoR1 digestion (Table 2). Two stages of PCR were used to amplify overlapping regions of the C-terminus of the AR cDNA. Kpn1/Psi1 or EcoR1 sense and antisense primers are upstream and downstream of the pSVAR Kpn1/Psi1 or EcoR1 sites, respectively. The mutation-specific primers were designed to contain the mutations to be incorporated into the AR cDNA. The first stage used 20 cycles to independently amplify the two fragments, upstream and downstream of the mutation. For the second stage, these products were size-separated on a 1% agarose gel and recovered using a QIAquick gel-extraction kit (Qiagen, Crawley, Surrey, UK). The purified fragments were then mixed in equal ratios and used as template for the fusion PCR stage (20 cycles), using Kpn1/Psi1 or EcoR1 sense and antisense primers. A thermostable, proofreading polymerase, PfuTurbo (Stratagene, La Jolla, CA, USA), was used. The fused PCR products were digested and the digestion products containing the mutation were then subcloned back into the digested WT pSVAR vector to generate a full-length AR cDNA containing the appropriate mutation. The sequences of the final mutant constructs were confirmed by sequencing. The reporter plasmid pGRE-LUC (androgen- and glucocorticoid-responsive firefly luciferase reporter vector) was a gift from Dr Axel Alléra. This reporter vector contains two consensus glucocorticoid response elements (GRE) and the TATA box (Knoke *et al.* 1999). The pRL-TK (Promega, Madison, WI, USA), a *Renilla* luciferase, was used as an internal control of transfection efficiency.

For studies using the mammalian two-hybrid system, the pM-LBD and the pVP16-rAR-(5–538) were generous gifts from Dr Jorma Palvimo (Ikonen *et al.*

1997; Moilanen *et al.* 1997). The pM-LBD expresses a Gal4 DNA-binding domain fusion of the WT hAR LBD (amino acids 624–919) and the pVP16-rAR-(5–538) expresses amino acid residues 5–538 of rat(r)AR. The point mutations were incorporated into the LBD using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and mutagenic primers as described in Table 2. The plasmid pCMX-UAS-TK-LUC (Tone *et al.* 1994) was used as a reporter and pRL-TK as an internal control for transfection efficiency. All PCR-amplified sequences of the mutated plasmid constructs were confirmed by direct sequencing.

Transactivation assay

The transactivation efficiencies of the mutated pSVARs, and WT AR were compared in transiently transfected COS-1 cells (ATCC, Manassas, VA, USA). Briefly, 2×10^5 COS-1 cells were transferred on to 12-well plates and cultured overnight with DMEM (Life Technologies, Paisley, UK) supplemented with penicillin 50 U/ml, streptomycin 50 µg/ml, glutamine 2 mM, MEM non-essential amino acids $1 \times$, and 10% (vol/vol) dialyzed EBS. The cells were transfected with 200 ng WT or mutated pSVAR, 500 ng pGRE-LUC, 5 ng pRL-TK and 2.1 µl Transfast per well as directed by the manufacturer. After 1 h, 0–100 nM (final concentrations) DHT was added. After incubation for 48 h the cells were lysed and assayed using a dual-luciferase assay system (Promega). The ratio of firefly to *Renilla* luciferase units was measured using a Turner TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA).

Figure 1 DHT-induced transactivation of the WT and mutated pSVARs. Each bar represents the mean $\pm$ S.E.M. from three independent experiments performed in triplicate; transactivation of the WT pSVAR with 100 nM DHT was adjusted to 1. Letters indicate level of significance (a = $P < 0.05$, b = $P < 0.01$, c = $P < 0.001$) compared with the transactivation of the WT pSVAR with the same DHT concentration, by Mann–Whitney U test. rLFU, relative Luciferase Firefly Units.

The mammalian two-hybrid system to study N-/C-terminal interaction

COS-1 cells (2×10^5) were transferred on to 12-well plates and cultured with 10% dialyzed fetal bovine serum and other supplements as described above. After 24 h, the cells were transfected with 250 ng WT or mutated pM-LBD, 250 ng pVP16-rAR-(5–538), 500 ng pCMX-UAS-TK-LUC and 5 ng pRL-TK as an internal control for transfection efficiency. Transfast at 3 μ l/ μ g transfected plasmid DNA was used as directed by the manufacturer. After 1 h, DHT to a final concentration of 10 nM was added. After 48 h incubation the cells were lysed and assayed using a dual-luciferase assay system as described above.

Statistical analysis

All data are expressed as means $\pm$ S.E.M. and all statistical analyses were performed using the Mann–Whitney U test. Differences at $P < 0.05$ were considered significant.

Results

The transactivational capacity of the mutated full-length ARs are given in Fig. 1. All the mutations had an effect on AR transactivation. Three mutations, D695N, R774H and L907F, presented with a near-complete loss

of ligand-dependent transactivation. Two mutations, Y763C and R855H, presented with moderate transactivation at supraphysiological (100 nM) concentrations of DHT. Q798E presented with good transactivational capacity with higher concentrations (≥ 10 nM) of the ligand.

The ligand-dependent N-/C-terminal interaction was relative to that of the WT AR only with one mutation, Q798E (Fig. 2). Some interaction was present also with the mutation R855H. All the other mutations, D695N, Y763C, R774C and L907F, led to a near-complete loss of N-/C-terminal interaction.

Figure 2 DHT (10 nM)-induced N-/C-terminal interaction of the WT and mutated pM-LBDs ((pM-LBD+pVP16-rAR)/pM-LBD). Each bar represents the mean $\pm$ S.E.M. from three independent experiments performed in triplicate; N-/C-terminal interaction of the WT LBD was adjusted to 1. Letters indicate level of significance (c = $P < 0.001$) compared with the N-/C-terminal interaction of the WT pM-LBD, by Mann–Whitney U test. rN/C interaction, relative N-/C-terminal interaction.

In general, the transactivational capacity and N-/C-terminal interaction of the mutated AR correlated well with the phenotype. D695N, R774H and L907F led to a loss of both transactional potential and N-/C-terminal interaction and the phenotype was CAIS. Q798E showed normal N-/C-terminal interaction and relatively well-preserved transactivational capacity, and the phenotype was PAIS.

However, there were two exceptions. Y763C presented with moderate transactivational capacity concordant with the phenotype of PAIS. However, the N-/C-terminal interaction was drastically diminished. R855H showed some transactivation and N-/C-terminal interaction but the phenotype was CAIS.

Discussion

To date, over 600 mutations in the AR have been reported, of these more than 300 have been associated with AIS. Furthermore, a majority of these mutations are missense mutations found in the LBD of the AR, see also McGill AR database (www.mcgill.ca/androgendb) and references therein (Gottlieb *et al.* 2004).

Interactions between different functional domains of the AR occur either directly by intra- or intermolecular interaction or via interaction with associated proteins that in turn affect other domains. Disrupted N-/C-terminal interaction due to mutations in the LBD is a known mechanism for the molecular abnormality in AIS. In the LBD, the region critical for N-/C-terminal interaction includes helices 4, 5, 6, and 12 as well as interhelical regions between helices 3 and 4 or 10 and 11 (Langley *et al.* 1998, He *et al.* 1999, Lim *et al.* 2000, Quigley *et al.* 2004, Thompson *et al.* 2001, Ghali *et al.* 2003). Our study shows that apart from these, regions just before helix 3, between helices 5 and 6, and at helix 10 are also important for this function.

In this study, we examined five naturally occurring AR gene mutations with normal ligand binding capacity (both $B_{\max}$ and K_d were within our normal range). Additionally, one mutation (Y763C) with decreased ligand binding affinity (K_d 3.0×10^{-10} mol/l) was selected. As N-/C-terminal interaction is dependent on high affinity ligand binding, with this mutation, disrupted N-/C-terminal interaction is most likely due to the abnormal binding of the ligand. Y763 lies between helices 5 and 6, the mutation not only almost uniformly associated with PAIS (Ahmed *et al.* 2000) but also as a somatic mutation in prostate cancer (Takahashi *et al.* 1995). As in our patient, this mutation leads to decreased androgen binding affinity, evidenced by high K_d in several reports. Substitution of tyrosine with histidine has been associated with a complete phenotype, no ligand binding studies have been performed with this mutation (Quigley *et al.* 1995).

Of the remaining five mutations, Q798E had no significant effect on N-/C-terminal interaction. Q798 is located at the C-terminal end of helix 7. Q798E is the only mutation reported at this position. The binding of the Q798E mutated AR has been found to be uniformly normal and the phenotype of the affected individuals are relatively mild, ranging from MAIS to PAIS with female sex of rearing (Bevan *et al.* 1996, Wang *et al.* 1998). This mutation has also been found as a genomic mutation in a patient with prostate cancer (Evans *et al.* 1996). Our present study was not able to reveal the principal mechanism of this mutation's pathogenicity.

With the remaining four AR mutations (D695N, R774H, R855H, and L907F) disruption of the N-/C-terminal interaction is a mechanism for diminished androgen action. D695 is located just before helix 3 at the N-terminal end of the AR LBD and three unique missense mutations at this position have been reported (Dork *et al.* 1998, Hiort *et al.* 1998). The aspartic acid residue at this position is well conserved and is known to be crucial for normal AR function: the CAIS phenotype has been reported with all three mutations (D695N, D695H and D695V). Substitution of aspartic acid with histidine leads to decreased ligand binding whereas substitution with asparagine does not disrupt ligand binding.

Two different substitutions (R774H and R774C) at position 774 have been associated with AIS and these both are relatively common (Prior *et al.* 1992, Ahmed *et al.* 2000). This site is of interest as substitution of arginine with cysteine ablates specific androgen binding activity but substitution with histidine preserves normal androgen binding capacity at 37 °C. However, thermolability is increased (Prior *et al.* 1992).

AR codon 855 at H10 is a mutational hot spot, 22 patients carrying a mutation at this position have been reported. Our patient with R855H had CAIS. This is in discordance with our results from the transactivation and N-/C-terminal interaction assays which both showed partial activity. Furthermore, in the literature this mutation has been associated with variable phenotypes, including PAIS. Thus, it is possible that our patient has other factors preventing normal masculinization during the fetal period. Elhaji *et al.* (2004) examined mutations R855C and R855H, and found that whereas R855C leads to very low binding of the ligand, R855H preserves ligand binding but is associated with diminished transactivation and N-/C-terminal interaction.

L907 is the last amino acid at H12 which is well known to be critical for N-/C-terminal interaction. Our patient is the only one reported to carry this mutation (Bevan *et al.* 1996).

He *et al.* (2002) reported that transactivation of prostate-specific antigen (PSA) and probasin enhancer/promoter regions is dependent on N-/C-terminal

Figure 3 Diagram showing the AR LBD crystal structure with a synthetic androgen R1881 (illustrated in green). PDB accession no. 1e3 g (Matias *et al.* 2000). The diagram shows the clusters of mutated residues reported to be critical for AR N-/C-terminal interaction. The five named residues were included in this study. Those in blue are in (or closely preceding) helix (H) 12, those in yellow are part of the ligand-binding pocket. The residues in cyan are at the top of the coactivator- or NTD-binding pocket. The pink residues (D695, R752, F754, Y763, and R774) seem to form a new region of the LBD that is important for N-/C-terminal interaction. The red residue is R855, which does not belong to any of these categories.

interaction whereas the sex-limited protein gene and mouse mammary tumor virus long terminal repeat do not require this interaction. The GRE promoter used by us was not included in their study. Our results show that transactivation of this promoter correlates well with AR N-/C-terminal interaction. Holterhus *et al.* (2002) have studied activation profiles of anabolic steroids, testosterone-precursors and virilizing androgens. They used three different androgen responsive promoter constructs, including GRE, and showed that different androgenic steroids led to different promoter activation profiles.

Recently, He *et al.* (2004) reported crystal structures of the ligand-activated AR LBD with and without bound FXXLF peptides. They identified key residues that establish motif binding specificity and concluded that residues M734, M894, E897, and K720 play a prominent role in AR FXXLF binding and undergo significant conformational change when in contact with this peptide.

The crystal model of the AR LBD shows that there are different clusters of mutations that interfere this action (Fig. 3). Mutations at or closely preceding helix 12 perturb co-activator or NTD binding. Another cluster are mutations at the top of the co-activator (NTD) binding pocket. A third group of mutations include those perturbing ligand binding affinity and perhaps the structure and/or stability of the LBD. Furthermore, the AR LBD crystal model shows that D695, Y763 and R774 together with some other mutations found to be important for N-/C-terminal interaction, form an interface that might come into contact with the AR NBD.

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authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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