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Variable Loss of Functional Activities of Androgen Receptor Mutants in Patients with Androgen Insensitivity Syndrome

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Key Words
Androgen insensitivity syndrome · Androgen receptor · Disorders of sex development · Functional assays · Molecular phenotype · Pathogenic mutations

Abstract
Androgen receptor (AR) mutations in androgen insensitivity syndrome (AIS) are associated with a variety of clinical phenotypes. The aim of the present study was to compare the molecular properties and potential pathogenic nature of 8 novel and 3 recurrent AR variants with a broad variety of functional assays. Eleven AR variants (p.Cys177Gly, p.Arg609Met, p.Asp691del, p.Leu701Phe, p.Leu723Phe, p.Ser741Tyr, p.Ala766Ser, p.Arg775Leu, p.Phe814Cys, p.Lys913X, p.Ile915Thr) were analyzed for hormone binding, transcriptional activation, cofactor binding, translocation to the nucleus, nuclear dynamics, and structural conformation. Ligand-binding domain variants with low to intermediate transcriptional activation displayed aberrant Kd values for hormone binding and decreased nuclear translocation. Transcriptional activation data, FxxFF-like peptide binding and DNA binding correlated well for all variants, except for p.Arg609Met, p.Leu723Phe and p.Arg775Leu, which displayed a relatively higher peptide binding activity. Variants p.Cys177Gly, p.Asp691del, p.Ala766Ser, p.Phe814Cys, and p.Ile915Thr had intermediate or wild type values in all assays and showed a predominantly nuclear localization in living cells. All transcriptionally inactive variants (p.Arg609Met, p.Leu701Phe, p.Ser741Tyr, p.Arg775Leu, p.Lys913X) were unable to bind to DNA and were associated with complete AIS. Three variants (p.Asp691del, p.Arg775Leu, p.Ile915Thr) still displayed significant functional activities in in vitro assays, although the clinical phenotype was associated with complete AIS. The data show that molecular phenotyping based on 5 different functional assays matched in most (70%) but not all cases.

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The androgen receptor (AR) is a ligand-dependent transcription factor and belongs to the superfamily of nuclear receptors [Laudet et al., 1999]. It is well-known that *AR* mutations can prevent normal development of both internal and external male structures [Quigley et al., 1995; Brinkmann, 2001]. End-organ resistance to androgens has been designated as androgen insensitivity syndrome (AIS) and is distinct from other XY disorders of sex development like 17β-hydroxy-steroiddehydrogenase type 3 deficiency or 5α-reductase type 2 deficiency [Wilson et al., 1993; Boehmer et al., 1999; Hughes, 2008]. The *AR* gene is located on the X chromosome and encodes a 110-kDa protein of 920 amino acid residues [Brown et al., 1989; Kuiper et al., 1989; Gottlieb et al., 2012 and www.androgendb.mcgill.ca]. Structural organization of the 8 coding exons is essentially identical to those of other steroid hormone receptors [Kuiper et al., 1989]. Also comparative structural and functional analysis of nuclear hormone receptors has revealed a common structural organization in 4 different functional domains: an NH_2-terminal domain (NTD) that harbors the major transcription activation functions and several structural subdomains [Jenster et al., 1995], a DNA-binding domain (DBD), a hinge region and a ligand-binding domain (LBD), of which detailed structural information has been published [Matias et al., 2000; Sack et al., 2001; Shaffer et al., 2004]. The 3-dimensional structure of the AR-LBD has the typical nuclear receptor LBD fold with a ligand-binding pocket consisting of 18 amino acid residues [Matias et al., 2000]. Upon interaction with hormone, the AR-LBD is folded as a sandwich of 11 α-helices and 2 antiparallel β-strands, resulting in a hydrophobic groove that interacts with LxxLL- and FxxLF-like motifs present in AR coregulators and also in the AR-NTD [He et al., 2000; Steketee et al., 2002; Dubbink et al., 2006].

Information of the sequence structure of the *AR* gene has facilitated the study of molecular defects associated with AIS. A large number of mutations in the *AR* has been identified [Gottlieb et al., 2012; www.androgendb.mcgill.ca]. The majority of mutations is located in the AR-LBD, less frequent are mutations in the AR-DBD, and AR-NTD mutations are very rare. *AR* mutations in X-linked AIS are associated with a variety of clinical phenotypes [Boehmer et al., 2001]. This provides the opportunity to correlate *AR* mutations with impairments of specific physiological functions. More fundamentally, the naturally occurring *AR* mutations are an important source for studies of protein structure-function relationships.

Although several reports have established the pathogenic nature of *AR* variants found in AIS individuals with different functional assays [Umar et al., 2005; Wong et al., 2008; Elfferich et al., 2009; Tadokoro et al., 2009], prediction of phenotypes on the basis of detected mutations can be difficult. To optimize molecular diagnosis, an extensive functional analysis of *AR* variants is desired. For counseling strategies and for future outcome predictions, a correct functional diagnosis is very important as well as for prognosis on the risks of gonadal malignancy [Cools et al., 2011]. A combination of different functional analyses, designed to test *AR* variants at different stages in AR functioning (e.g. hormone binding, translocation to the nucleus, nuclear dynamics, cofactor binding, and transcriptional activation), will provide a more accurate prediction of AR functioning and will help to establish a more exact phenotypic characterization.

The aim of the present study was to determine the molecular properties and potential pathogenic nature of 8 novel *AR* variants of unknown clinical significance and of 3 previously reported variants. The outcome of these studies will contribute to optimal treatment of the patients and counseling strategies of relatives. In addition, data from this study provides new information on AR structure-function relationship.

Materials and Methods

Patients

The AIS patient samples were provided by different clinical centers in the Netherlands, Belgium and Indonesia, and all sequence variants were identified at the Department of Clinical Genetics, Erasmus Medical Center Rotterdam, The Netherlands. Informed consent was obtained from patients and/or their parents at each center.

Mutation Analysis

Extraction of DNA from peripheral blood cells was performed according to standard techniques. The coding exons and exon/intron boundaries of the *AR* gene were analyzed by direct sequencing on an ABI3730XL automated sequencer (Applied Biosystems, Foster City, Calif., USA). All mutations were confirmed by a duplicate sequencing experiment on the patients' DNAs.

The positions of the different mutations (p.Cys177Gly, p.Arg609Met, p.Asp691del, p.Leu701Phe, p.Leu723Phe, p.Ser741Tyr, p.Ala766Ser, p.Arg775Leu, p.Phe814Cys, p.Lys913X, and p.Ile915Thr) are schematically indicated in figure 1A–C. The numbering of the amino acid residues is according to the National Center for Biotechnology Information (NCBI) reference sequence number NM_000044.2, which refers to a gene size of 187,246 nucleotides and an AR protein of 920 amino acid residues with a polyglutamine tract of 23 and a polyglycine tract of 23 [Gottlieb et al., 2012; www.androgendb.mcgill.ca]. Nucleotide numbering starts at the A of the ATG start codon, according to nomenclature of the Human Genome Variation Society.

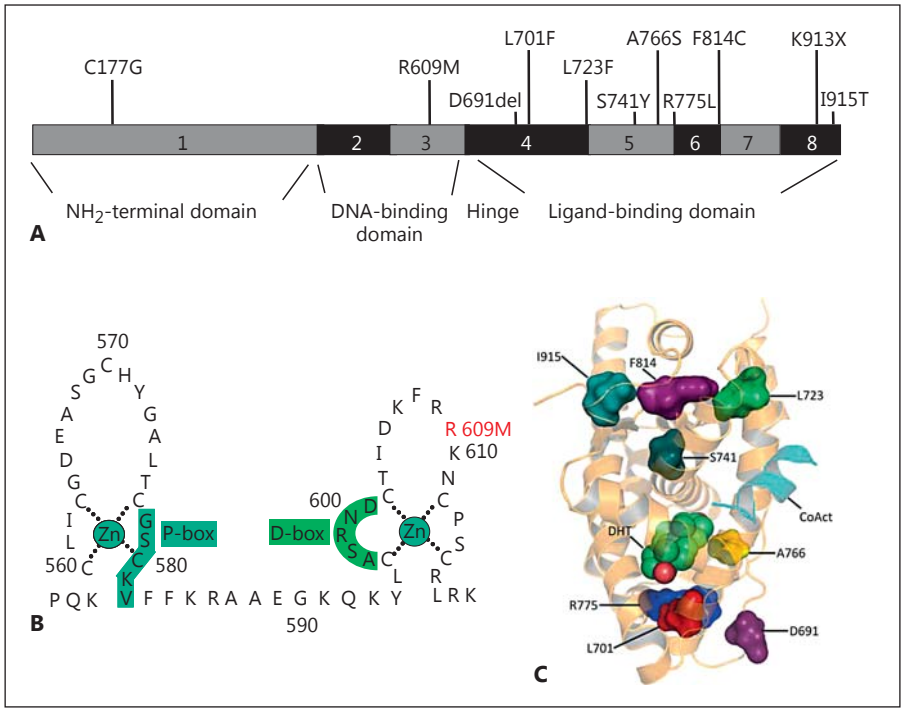

Fig. 1. A Schematic overview of the localization of the different mutations in the AR protein. Numbering is according to the NCBI reference sequence number NM_000044.2. The international 1-letter code is used for amino acid residues. X represents a premature stop, while del indicates a deletion of an amino acid residue. **B** DNA-binding domain of the AR with the location of the p.R609M mutation present in the second zinc cluster. **C** Positions of the mutated residues in the LBD of the AR (with exception of p.K913X) are represented in a 3D model based on X-ray crystallographic data of the AR LBD complexed with a reference ligand.

One of the 11 variants (p.Cys177Gly) was found in an Indonesian male partial androgen insensitivity (PAIS) patient and 1 AR-DBD variant (p.Arg609Met) was found in a complete androgen insensitivity (CAIS) patient, whereas the remaining variants were localized in the AR-LBD and found in CAIS and PAIS patients. Three variants have been reported previously: p.Asp691del in a CAIS patient of whom the mother was a carrier; p.Leu723Phe in a female PAIS patient and p.Ala766Ser in a male PAIS patient of whom the mother was also carrier [Hiort et al., 1996; Chávez et al., 2001; Tadokoro et al., 2009]. Further information on phenotypes, family history and gender identities are summarized in table 1.

Recombinant Plasmids

Human wild type *AR* (wt *AR*) cDNA expression plasmid pSVAR0 was used to generate constructs encoding mutant *AR*s using QuickChange site-directed mutagenesis (Stratagene, La Jolla, Calif., USA). *Asp*718 and *Bam*HI fragments of mutant *AR*s were exchanged with wild type fragments in pSVAR0 with the exception of AR p.Cys177Gly, where *Afl*II and *Hin*dIII were applied. Most GFP-tagged mutant AR expression constructs were generated by subcloning of the mutated fragments into pEGFP-AR0 by *Eco*RI and *Hin*dIII digestions [Farla et al., 2004]. The AR p.Cys177Gly fragment was subcloned by digestion with *Afl*II and *Asp*718. Mutants encoding p.Phe814Cys, p.Lys913X or p.Ile915Thr were generated using site-directed mutagenesis on pEGFP-AR0. All constructs were confirmed by direct sequencing. The Gal4-DBD-AR FxxLF, the Gal4-DBD-Gelsolin FxxFF and the Gal4-DBD-SRC -3box1 LxxLL peptide expression constructs have been described previously [Dubbink et al., 2004; Van de Wijngaart et al., 2006, 2012].

Western Blot Analysis

For Western blot analysis, Hep3B cells (1.5×10^4 cells per plate) were seeded in 3 × 6-well plates (Cellstar, Greiner, Frickenhausen, Germany) in αMEM (Lonza BioWhittaker) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 5% FCS (HyClone, Thermo Fisher Scientific). Next day the cells were washed with PBS, and the medium was substituted by medium containing 5% dextran-coated-charcoal-treated (DCC)-FCS (HyClone, Thermo Fisher Scientific) containing 1 nM synthetic androgen R1881. Four hours after addition of R1881, the cells were transfected with AR expression constructs using FuGENE6 reagent (Roche Diagnostics, Basel, Switzerland). Forty-eight hours after transfection, 150 μl ice-cold SDS Laemmli sample buffer containing 10 mM dithiotreitol (DTT) was added to each well. The cell lysates were transferred to 1.5-ml Eppendorf tubes and boiled for 2 min. After a short sonication step, 8 μl of the lysate was run on a 4–12% precast SDS-polyacrylamide gel (Criterion XT Biorad, Hercules, Calif., USA). Proteins were separated and blotted to a nitrocellulose transfer membrane (Protean, Whatmann, Dassel, Germany). Immunoblotting was performed using polyclonal antibody F39.4.1 [Van de Wijngaart et al., 2010]. Proteins were visualized via the Super signal Pico chemiluminescent substrate method (Thermo, Rockford, Ill., USA).

Androgen-Binding Assays

COS-1 cells (American Type Culture Collection; 1.75×10^6 cells per plate) were seeded into 15-cm^2 plates in DMEM containing 2 mM glutamine and 10% DCC-FCS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. After 24 h, the medium was exchanged for OptiMEM I medium (Invitrogen). Using Lipofectin (Invitrogen), the cells were then transfected with either 8.5 μg pSVAR0 or AR mutant. Sixteen hours post-transfection, the

Table 1. Overview of clinical phenotypes of AIS patients, their mutations and gender identities

Phenotypes[a]	Diagnosis	Quigley stage[b]	Social sex	Mutation	Nucleotide change	Location mutation	Previously described	Family investigations	
								heterozygous carriers	other members affected
PAIS									
P1	penoscrotal hypospadias	3	M	p.C177G	c.529T>G	exon 1	no	no information	
P2		6	F	p.L723F	c.2169G>T	exon 4	Hiort et al., 1996	mother	
P3			M	p.A766S	c.2296G>T	exon 5	Chavez et al., 2001	mother	
P4	coronal hypospadias		M	p.F814C	c.2441T>G	exon 6	no		
CAIS									
C1			F	p.R609M	c.1826G>T	exon 3	no		
C2	testes present	5	F	p.D691del	c.2071-2073del-CAG	exon 4	Tadokoro et al., 2009	mother	
C3	testes present	7	F	p.L701F	c.2103G>T	exon 4	no	mother	maternal aunt (CAIS)
C4	difficult to visualize testes	7	F	p.S741Y	c.2222C>A	exon 5	no	mother	
C5	inguineal hernia, testes present	7	F	p.R775L	c.2324G>T	exon 6	no	mother	
C6	dysgenetic testes with Sertoli cell adenomas	7	F	p.K913X	c.2737A>T	exon 8	no	no information	
C7	gonadectomized	7	F	p.I915T	c.2744T>C	exon 8	no	no information	

[a] P1–P4 = PAIS patients 1–4; C1–C7 = CAIS patients 1–7. [b] Quigley et al., 1995.

cells were transferred to DMEM/FCS. Following further 24 h incubation, the harvested cells were used in a whole cell-binding assay with ^{3}H-labeled mibolerone (Perkin Elmer), as previously described [Bevan et al., 1996]. Scatchard analysis was performed using the Combicept 2000 Steroid Receptor Assay Software (Packard) that provided the binding site dissociation constant Kd.

Transactivation Assays

For transcription activation studies, Hep3B cells (5×10^4 cells per plate) were cultured in αMEM medium supplemented with 5% FCS, 100 U/ml penicillin, 2 mM glutamine and 100 μg/ml streptomycin in 24-well plates (Greiner Bio). After 24 h, αMEM supplemented with 5% FCS was replaced by αMEM with 5% DCC-FCS supplemented with a range of 10 pM–100 nM synthetic androgen R1881. Four hours after addition of R1881, the cells were transfected with 50 ng AR expression construct and 100 ng ARE_2-TATA-Luc reporter plasmid using FuGENE6 reagent. The cells were lysed 24 h after DNA transfection, and luciferase activity was measured in a luminometer (GlowMax Luminometer, Promega).

Coactivator Binding

For coactivator interaction assays, the same procedure of cell culture was used as described for transcriptional activation assays in presence of 1 nM R1881. A mixture of 50 ng AR expression construct, 50 ng Gal4-DBD-AR FxxLF, Gal4-DBD-Gelsolin FxxFF or Gal4-DBD-SRC3box1 LxxLL expression construct, 150 ng (UAS)4-TATA-Luc reporter plasmid and 1 μl FuGENE was applied per well [Van de Wijngaart et al., 2006]. The cells were lysed 24 h after transfection, and luciferase activity was measured in a luminometer.

Quantitative Live-Cell Imaging of the Subcellular Distribution

Two days prior to microscopic analysis, Hep3B cells were grown on glass cover slips in 6-well plates in αMEM (Cambrex) supplemented with 5% DCC-FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. At least 4 h before transfection, the medium was substituted by medium containing 5% DCC-FCS. Cells were transfected with 1 μg GFP-AR expression construct in FuGENE transfection medium (Roche). Four hours after transfection, the medium was replaced by medium with 5% DCC-FCS with the indicated concentration of R1881.

Live-cell imaging was performed using a confocal laser-scanning microscope (LSM510; Carl Zeiss MicroImaging, Inc.) equipped with a Plan-Neofluar 40×/1.3 NA oil objective. Enhanced green fluorescent protein (EGFP) was excited using a 488-nm argon laser line at moderate laser power, and emission was detected using a 505–530-nm bandpass emission filter to obtain images for subcellular localization analysis. Fluorescence intensities were measured in a region of the nucleus and a region just outside the nucleus of the cells, and the relative nuclear intensity was determined using the equation $I_{rel\text{-}nuc} = ((I_{nuc} - I_{bg})/(I_{nuc} - I_{bg}) + (I_{cyt} - I_{bg}))$, where I_{bg} is the background signal.

Fluorescence Recovery after Photobleaching (FRAP)

Sample preparation for strip-FRAP was similar as used for imaging of the subcellular distribution. Strip-FRAP analysis was performed with a similar configuration as described by Van Royen et al. [2007, 2009]. In short, fluorescence in a narrow strip (1 μm – corresponding to 10 pixels) spanning the entire nucleus was bleached, and the recovery of fluorescence inside this strip was monitored in time with a 100-ms interval using low laser power. Fluorescence intensity in the strip was expressed relative to pre-

bleach intensities ($I_{prebleach}$) and the intensity directly after bleaching (I_0): $I_{norm,\,t} = (I_t - I_0)/(I_{prebleach} - I_0)$. AR mutant dynamics was directly compared with wt AR and a DNA-binding deficient mutant (p.Ala574Asp) [Farla et al., 2004].

Structural Analysis of AR Mutants

Amino acid residue substitutions were done in a proprietary wt AR structure bound to 5α-dihydrotestosterone (DHT). Each mutation has been minimized in Yasara using the Yamber2 force field and visually inspected [Krieger et al., 2004]. Sequence and structure alignments were based on the NuclearRDB [Horn et al., 2001]. All pictures were created in PyMOL (PyMOL Molecular graphics system, Version 1.2r3pre, Schrödinger, LLC).

Results

Protein Expression Studies and Transcriptional Activity

Western blot analysis of transiently transfected AR mutants shows that the expressed proteins are of the correct molecular size (fig. 2A). Overall negative results in functional assays for some of the mutants cannot be explained by the variation of expression level of the mutant proteins.

AR variants identified in PAIS patients (p.Cys177Gly, p.Leu723Phe, p.Ala766Ser, and p.Phe814Cys) induced transcriptional activities between 12 and 81% of wild type activity at 1 nM R1881 in an ARE_2-TATA-Luc reporter assay (fig. 2B). For the p.Leu723Phe mutant, a considerable increase in induced activity was observed at 10 nM R1881. The p.Phe814Cys mutant, identified in a PAIS patient, displayed an activity of ~20% at both 1 and 10 nM R1881. These results are in good agreement with the observed PAIS phenotype.

Considerable activities were also found for 3 variants identified in clinically CAIS patients (p.Asp691del, p.Arg775Leu and p.Ile915Thr) at 10 nM R1881; even at 0.1 nM R1881 some activity can be measured for p.Asp691del and p.Ile915Thr (fig. 2C). For 4 other mutants identified in CAIS patients (p.Arg609Met, p.Leu701Phe, p.Ser741Tyr, and p.Lys913X) no transcriptional activity was detectable, even at high hormone concentrations (fig. 2C) confirming and supporting the clinical findings with respect to the phenotype (CAIS).

Hormone-Binding Characteristics

Scatchard plot analysis with ^{3}H-mibolerone as synthetic radioactive androgen was performed on a selection of mutants that displayed some activity in the transcriptional activation assays (e.g. p.Arg775Leu, p.Phe814Cys, p.Ile915Thr) and the previously reported mutants p.Leu-

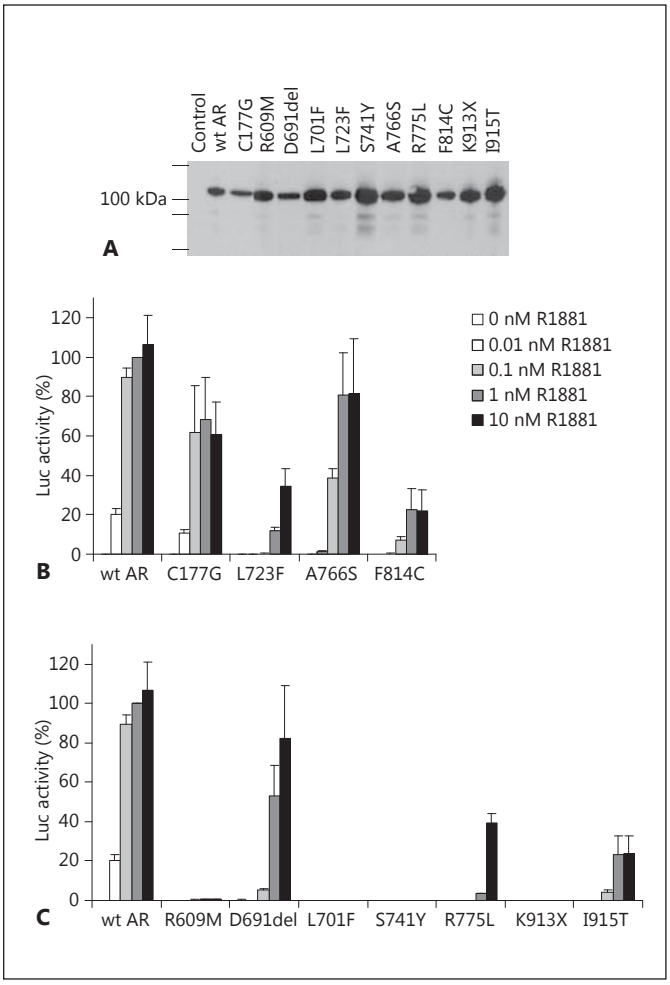

Fig. 2. A Western blot analysis of AR mutants. Wild type AR (wt AR) and mutants were expressed in Hep3B cells in the presence of 1 nM R1881. In addition, a cellular extract of Hep3B cells without transfected AR was included as a negative reference (control). **B**, **C** Transcriptional activation assays in Hep3B cells. Dose response curves of wt AR, p.C177G, p.L723F, p.A766S and p.F814C (**B**) and wt AR, p.R609M, p.D691del, p.L701F, p.S741Y, p.R775L, p.K913X, and p.I915T (**C**) in the presence of increasing concentrations of R1881 (0–10 nM). The activity of wt AR at 1 nM R1881 was set at 100% and the other data points were calculated relative to that. Values represent the means ± SEM of at least 3 experiments, each performed in triplicate.

723Phe and p.Ala766Ser [Hiort et al., 1996; Chávez et al., 2001] (table 2). A normal Kd value can be expected for mutants p.Cys177Gly and p.Arg609Met. The Kd value for the p.Asp691del mutant was published previously [Tadokoro et al., 2009]. All mutants with some transcriptional activation displayed Kd values that were significantly higher than that of the wild type receptor, indicat-

Table 2. Summary of functional studies performed on the different AR mutants

Mutation	AR domain	Patients[a]	% Transcription activation[b]	Kd nM ± SD[c]	C/N distribution[b]	DNA binding[b]	% Peptide binding[b, d]	Published phenotype [ref.]
wt AR			100	0.40±0.09	C<<N	++	100	
p.C177G	N-term	P1	68	n.m.	C<<N	++	52	novel
p.L723F	LBD	P2	12	1.72±0.35	C=N	+/–	27	CAIS [Hiort et al.,1996]
p.A766S	LBD	P3	81	0.91±0.24	C<<N	+	59	PAIS [Chávez et al., 2001]
p.F814C	LBD	P4	23	1.22±0.29	C<<N	+	33	novel
p.R609M	LBD	C1	0	n.m.	C<<N	–	90	novel
p.D691del	LBD	C2	53	1.06[e]	C<<N	+/–	60	PAIS [Tadokoro et al., 2009]
p.L701F	LBD	C3	0	n.m.	C>>N	–	2	novel
p.S741Y	LBD	C4	0	n.m.	C>>N	–	1	novel
p.R775L	LBD	C5	3	0.93±0.68	C<N	–	30	novel
p.K913X	LBD	C6	0	n.m.	C>>N	–	1	novel
p.I915T	LBD	C7	23	1.23 ± 0.49	C<<N	+	24	novel

C/N = Cytoplasmic/nuclear distribution; LBD = ligand-binding domain; n.m. = not measured; N-term = NH_2-terminal domain; – = no DNA binding; ++ = DNA binding; + = reduced DNA binding; +/– = intermediate DNA binding.

[a] P1–P4 = PAIS patients 1–4; C1–C7 = CAIS patients 1–7 (see table 1). [b] In the presence of 1 nM R1881. [c] Kd values were calculated from the negative inverse of Scatchard plots. Kd values (in nM) are given as the means ± SD of at least 3 independent binding assays performed in COS-1 cells with ^{3}H-mibolerone. [d] FxxFF motif peptide. [e] Tadokoro et al., 2009.

ing a decreased affinity for mibolerone, although the Kd value did not directly correlate with transcriptional activity in a quantitative sense.

Intracellular Distribution and Nuclear Dynamics

Intracellular distribution using GFP-tagged AR mutants was investigated in living cells in the presence of different R1881 concentrations (fig. 3A, B). In addition, their nuclear mobility at 1 nM R1881 was studied using FRAP technology and compared to wt AR that shows a transient immobilization due to DNA binding (positive control), and a DNA-binding deficient AR mutant (p.Ala574Asp; negative control) which lacks this transient immobilization, as is shown in several previous studies (fig. 4A–K) [e.g. Farla et al., 2004; Van Royen et al., 2009]. The transcriptionally active mutants p.Cys177Gly, p.Asp691del, p.Ala766Ser, p.Phe814Cys, and p.Ile915Thr all displayed nuclear localization similar to that of the wt AR (fig. 3A, B; as typical examples only p.Phe814Cys and p.Ile915Thr are shown). Two mutants with minimal transcriptional activation activities were either mainly nuclear (p.Arg775Leu) or distributed equally between cytoplasm and nucleus (p.Leu723Phe; fig. 3A, B) in the presence of 1 nM R1881. Only in the presence of 100 nM R1881, a maximal nuclear translocation was observed for these mutants, which could explain their minimal transcriptional activation activities (fig. 3A, B; only p.Leu723Phe is shown).

In comparison with wt AR (showing transient immobilizations) and a non-DNA-binding mutant (Ala574Asp; only freely mobile), the mutants p.Asp691del, p.Leu723Phe, p.Ala766Ser, p.Phe814Cys, and p.Ile915Thr showed an intermediate immobilization in the FRAP experiments, but Asp691del and Leu723Phe were more mobile than the other 3 mutants (fig. 4C, E, G, I and K, respectively). p.Cys177Gly displayed wt AR immobilization (fig. 4A). This is in contrast to p.Arg775Leu (fig. 4H) that lacked completely nuclear transient immobilization.

Transcriptionally inactive p.Leu701Phe, p.Ser741Tyr and p.Lys913X displayed a predominantly cytoplasmic localization, even at a high R1881 concentration (10 nM), whereas the transcriptionally inactive DBD mutant p.Arg609Met was mainly nuclear (results not shown). The homogeneous nuclear distribution of p.Arg609Met, unlike the speckled distribution as found for wt AR (fig. 3A), correlated well with the lack of immobilization measured by FRAP, similar to the previously published non-DNA-binding mutant p.Ala574Asp (fig. 4B) [Farla et al., 2004; Van Royen et al., 2007]. Similarly, the small nuclear fractions of p.Leu701Phe, p.Ser741Tyr and p.Lys913X displayed a rapid redistribution pattern after photobleaching, indicative of absence of nuclear immobilization (fig. 4D, F and J, respectively). In these mutants, the results reflected the absence of ligand activation, indirectly resulting in the inability to stably bind to DNA, where-

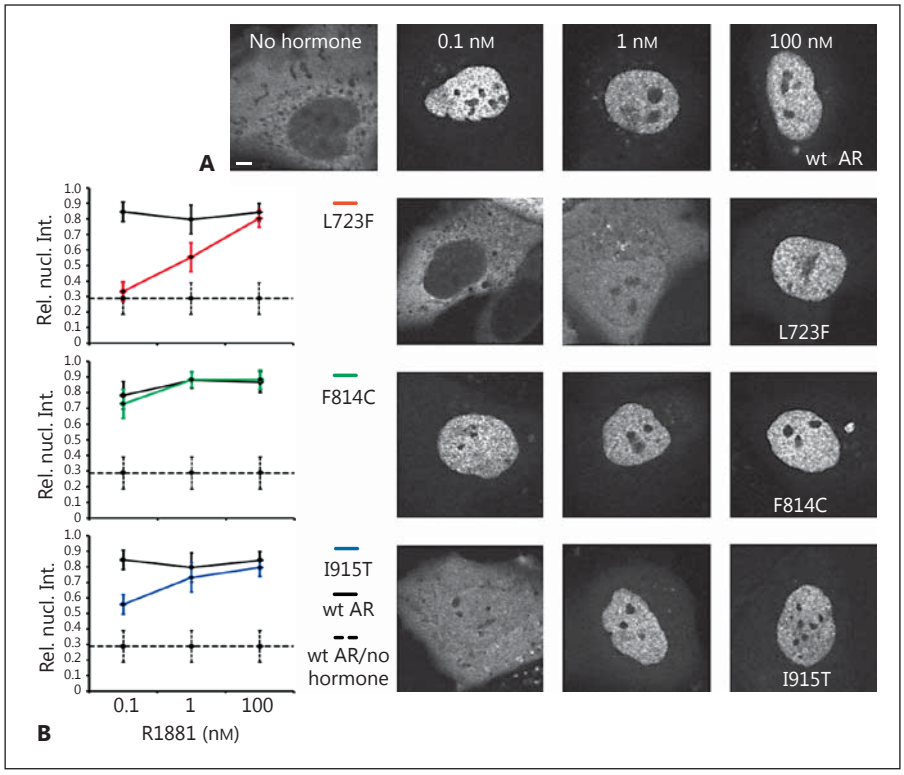

Fig. 3. A High-resolution confocal image analysis of Hep3B cells expressing GFP-tagged wt AR and AR mutants p.L723F, p.F814C and p.I915T. **B** In the graphs, the relative nuclear intensity is plotted at 3 different R1881 concentrations: 0.1, 1 and 100 nM, and the corresponding confocal images. In each graph also the wt AR is plotted.

as the p.Arg609Met substitution still enabled ligand binding and nuclear translocation but directly inhibited stable DNA binding. These properties were in accordance with the lack of transcriptional activation of the 4 mutants.

Interactions with an AR Cofactor-Derived α-Helical FxxFF Peptide Motif

A functional peptide one-hybrid interaction assay was designed as described previously [Van de Wijngaart et al., 2006]. Briefly, Hep3B cells were transiently transfected with constructs expressing a Gal4-DBD-FxxFF fusion protein (interaction motif present in the context of the AR coactivator gelsolin) and full-length AR mutants together with an (UAS)4-TATA-Luc reporter construct and incubated either in the absence or presence of 1 nM R1881. For all transcriptionally inactive mutants, insignificant peptide interactions were observed with the exception of the DBD mutant p.Arg609Met, which displayed wild type peptide interaction (fig. 5B). All novel PAIS mutants and the published PAIS mutants p.Asp691del and p.Ala766Ser displayed a relatively high peptide interaction (fig. 5A, B). These results correlated well with the transcriptional activation data. Remarkably, the novel CAIS variants p.Arg775Leu and p.Ile915Thr displayed significant activities in both transcriptional activation and peptide interaction (figs. 2C, 5B). Similar effects of the mutations on peptide interactions were found using the FxxLF motif of the AR-NTD and the LxxLL motif of SRC3box1 (results not shown).

Structural Analysis of AR Mutants

Structural and functional consequences of the various LBD variants can be predicted based on computer modeling of the mutant AR LBDs complexed with a reference ligand. In the AR-LBD modeling, the Leu723 residue is in close proximity to Lys721, which is one side of the charge clamp crucial for recognition and binding of cofactor proteins (fig. 1C). Changing the size of residue 723 to a larger residue, like phenylalanine in mutant p.Leu723Phe, prevents the optimal arrangement of Lys721 and explains the drop in peptide binding and transcriptional activation.

Substitution of Phe814 by a cysteine increases the space available in the small hydrophobic pocket, allowing a deeper burying of the Leu723 side-chain into the protein and altering the orientation of Lys721 with a concomitant negative effect on transcriptional activation (fig. 1C). Ile915 is in close contact with Phe814, forming

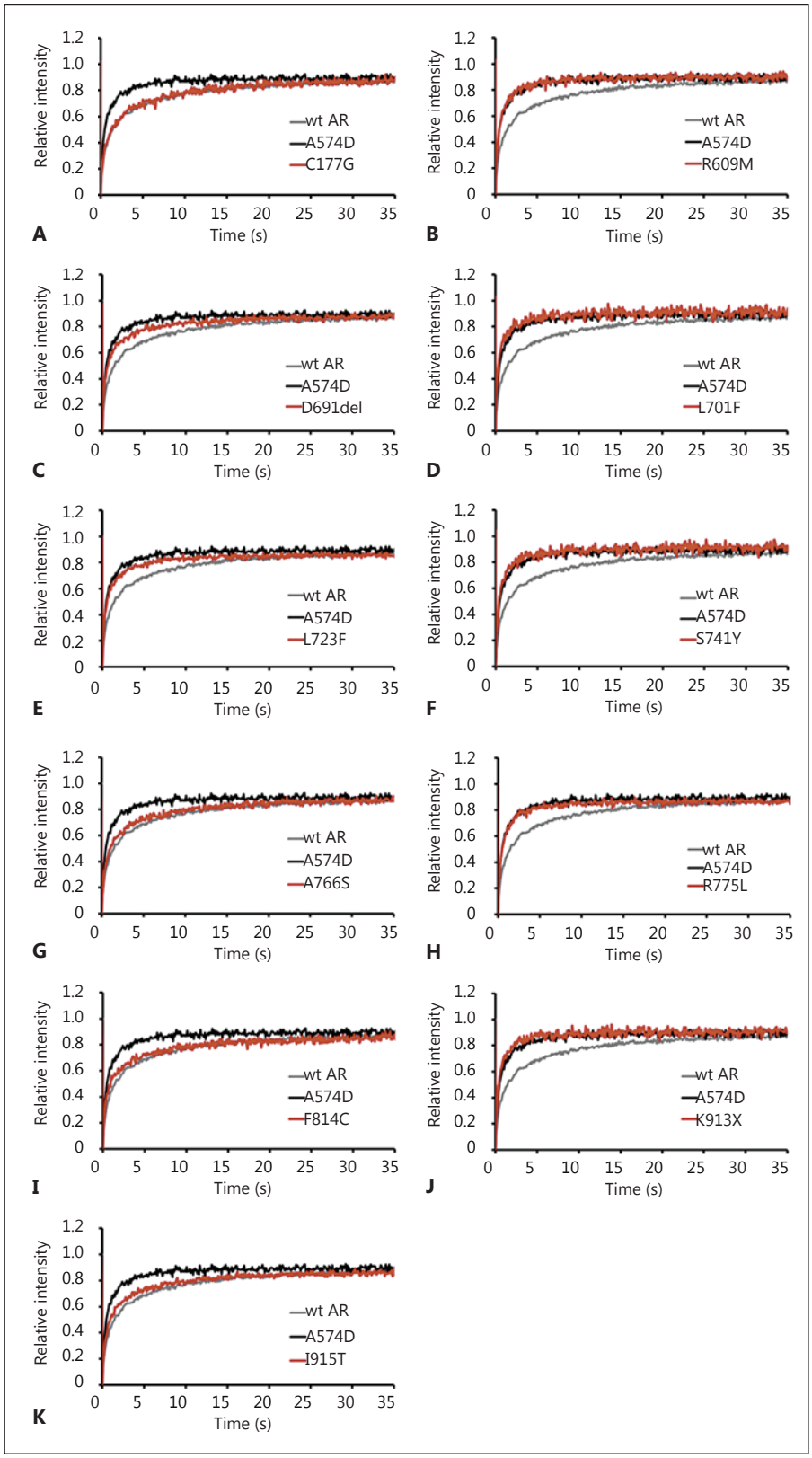

Fig. 4. Strip-FRAP analysis of Hep3B cells expressing GFP-tagged AR mutants p.C177G (**A**), p.R609M (**B**), p.D691del (**C**), p.L701F (**D**), p.L723F (**E**), p.S741Y (**F**), p.A766S (**G**), p.R775L (**H**), p.F814C (**I**), p.K913X (**J**), and p.I915T (**K**). FRAP analysis of the mutants (red curves) was performed in the presence of 1 nM R1881. Redistributions of wild type AR (grey curves) and a non-DNA-binding mutant (A574D) (black curves) were plotted as references.

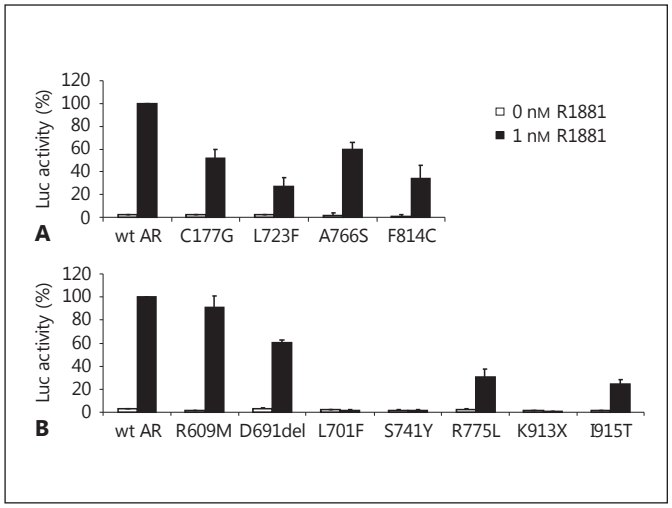

Fig. 5. A, **B** Interaction assay of an FxxFF-motif peptide as measured in Hep3B cells transfected with either wild type AR or AR mutant constructs. The gelsolin FxxFF motif peptide construct and the (UAS)4-TATA-Luc reporter plasmid were used in this interaction assay. Interaction was measured in the absence (open bars) and presence of 1 nM R1881 (filled bars). The interaction of wt AR at 1 nM R1881 was set at 100% and the other data points were calculated relative to that. Values represent the means ± SEM of at least 3 experiments, each performed in triplicate On top of the bars SD is displayed.

a direct bridge across the width of the LBD (fig. 1C). Substitution of isoleucine by threonine introduces a polar group, affecting Phe814 and consequently transcriptional activation.

Ser741 is located at the very heart of the AR-LBD. Given its close proximity to Phe814 and the ligand-binding pocket, it is postulated that in such a tightly packed space, where small residues are so conserved, a Ser741Tyr substitution will have a profound effect on structural integrity of the LBD (fig. 1C).

Helix-2 is in the AR-LBD replaced by a long loop of moderately ordered residues. Arg775 plays an important role in stabilizing this portion of the structure and forms H-bonds to Asn692 and Asp696, and perhaps to His690, at the bottom of the loop. Substitution of arginine by a leucine removes all 3 stabilizing H-bonds, explaining the resulting reduction in transcriptional activation.

Deletion of aspartic acid at position 691, which is located in the loop between helix 1 and helix 3, will likely alter orientation of either, or both, His690 and Asn692 with a resulting negative effect on this important stabilizing region and consequently on transcriptional activation.

Replacing Leu701 by a phenylalanine will inevitably result in a clash with Arg775 and Asp696, immediately destroying domain stabilizing interactions between these 2 residues, resulting in a total loss of transcriptional activation of mutant p.Leu701Phe.

Structurally, it is difficult to hypothesize why moderate loss of transcriptional activation results from the Ala766Ser mutation that would appear to be well-tolerated. In this mutant, a new H-bond is introduced to Asp768, and perhaps this new H-bond interferes with some required, but until now unidentified, flexibility.

Discussion

In the present study, molecular properties and the potential pathogenic nature of 8 novel and 3 previously identified AR variants are presented. Functionality of the mutant receptors was assessed with a variety of assays reflecting the spectrum of AR properties in the mechanism of action of androgens (table 2; fig. 6). Briefly, after ligand binding, the receptor is translocated to the nucleus, and the hormone-receptor complex binds specifically to binding sites on the chromatin. DNA binding is accompanied and stabilized by cofactors which bind via specific interaction motifs to the receptor. The AR-LBD preferentially interacts with cofactors that contain FxxLF motifs [Dubbink et al., 2004; Van de Wijngaart et al., 2006]. This finally results in transcriptional activation and/or suppression of specific androgen responsive genes and ultimately in development of a male phenotype. Thus, the identification and description of a defect in one or multiple steps could assist in understanding the observed clinical phenotypes in AIS patients [Brüggenwirth et al., 1998; Umar et al., 2005; Wong et al., 2008; Elfferich et al., 2009; Tadokoro et al., 2009; Orr et al., 2010].

At least 3 mutants (p.Leu701Phe, p.Ser741Tyr and p.Lys913X) directly and 2 mutants (p.Asp691del and p.Arg775Leu) indirectly interfered at the hormone-binding step in the cascade of androgen action (fig. 6). This is reflected by a lack of nuclear translocation and transcriptional activation combined with reduced cofactor peptide binding at physiological hormone concentrations (nM range). Four mutations (p.Leu723Phe, p.Ala766Ser, p.Phe814Cys, and p.Ile915Thr) have a more specific effect on cofactor peptide binding. Arg609Met directly interferes with stable DNA binding, and for p.Cys177Gly it may be speculated that the mutation directly or indirectly affects interaction with cofactors that interact with the NTD.

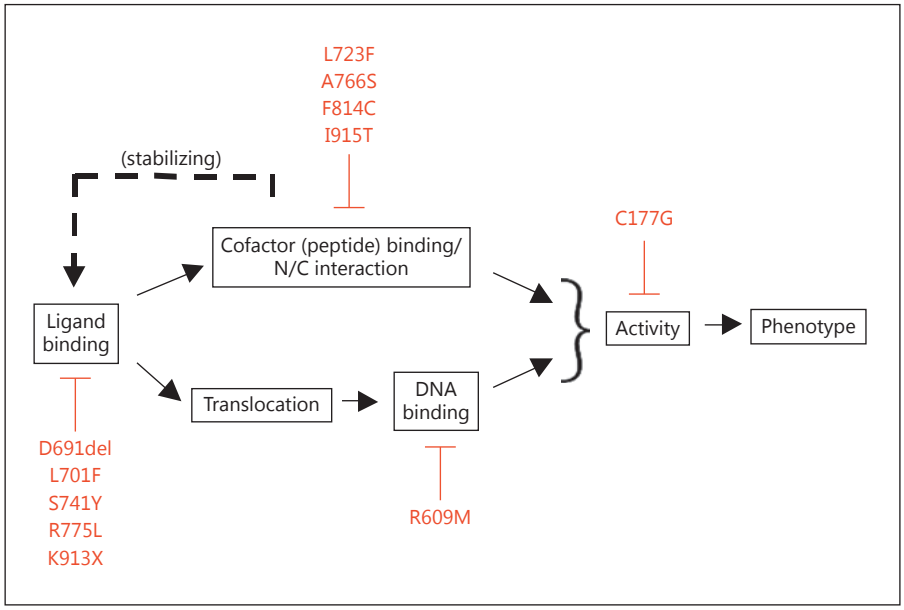

Fig. 6. Schematic representation of the cascade of steps in androgen action involving the androgen receptor. The sites which are being affected by the different AR mutations based on the outcome of the functional studies are indicated.

The data as summarized in table 2 can be clustered into 3 groups of patients based on clinical and biochemical phenotypic appearance. Firstly, a group of patients, designated as CAIS and with a so-called 'dead receptor' in functional assays: no transcriptional activation, no DNA binding and predominantly cytoplasmic localization (patients C1, C3, C4 and C6). Secondly, a group of phenotypically male PAIS patients (patients P1, P3 and P4): the mutations found in the PAIS boys displayed moderate but significant activities in all assays (table 2). Thirdly, a group of patients (patients P2, C2, C5 and C7) that were identified as phenotypically associated with CAIS [Hiort et al., 1996 and present study], but according to the data set of functional assays it can now be concluded that moderate (P2 and C5) to considerable (C2 and C7) functional activities can be attributed to these mutant receptors.

For some AR mutants in the first group (e.g. p.Leu701Phe, p.Ser741Tyr, p.Lys913X), no ligand-binding data were generated. These mutants displayed a complete absence of activity in 4 different assays, even at relatively high hormone concentrations. Obviously, adding hormone binding would have been more complete. However, in the indicated cases this extra information is of limited value, because these mutants do not enter the nucleus (table 2), and as a consequence, even in the presence of non-physiological high hormone concentrations, these mutants are completely inactive. Moreover, the structural predictions indicate a highly unstable LBD. DBD mutant p.Arg609Met found in a CAIS patient displayed no transcriptional activation and according to the FRAP data is not able to stably bind to DNA.

The second group of AR mutants found in the 3 phenotypically male PAIS patients (patients P1, P3 and P4) displayed a transient immobilization similar to wild type receptor. Therefore, a defective DNA binding seems not the cause of the observed partial transcriptional activation. These AR mutants are able to translocate efficiently to the nucleus even at low hormone concentrations. And, interestingly, transcriptional activation by F814C did not increase with increased ligand concentration (1 nM to 10 nM R1881), suggesting that a defective ligand binding is not the main problem in this mutant.

The novel mutation in the NTD (p.Cys177Gly) displayed partial activities in coactivator binding and transcriptional activation, and full potential for DNA binding. Pathogenic mutations in the N-terminal domain are very rare in AIS [Gottlieb et al., 2012; www.androgendb.mcgill.ca]. Almost all are nonsense mutations or deletions/insertions. An amino acid substitution like in the p.Cys177Gly case is novel and unique.

A third group of mutants (p.Asp691del, p.Arg775Leu, p.Leu723Phe, and p.Ile915Thr) consists of AR variants that all displayed an elevated Kd, a distorted LBD structure and partial activities for transcriptional activation, cofactor peptide binding, nuclear translocation, and dynamics. The AIS individual with the p.Asp691del mutation was reported earlier with a PAIS clinical phenotype [Tadokoro et al., 2009]. This is an interesting phenome-

non. Most likely, additional parameters like properties or expression of coactivators, which were not studied in the present investigation, can affect the phenotypic expression of a mutant AR. This can particularly play a role in those cases with the same AR mutation in different AIS individuals where a different clinical phenotype has been reported. However, variables like hormone metabolism and even not yet identified mechanisms can not be excluded. Investigation of this aspect will be highly relevant.

Comparing the data from table 2, it is evident that p.Ile915Thr and p.Phe814Cys are almost identical in all tests; yet it remains unclear why the clinical phenotypes are so different.

The individual with a p.Leu723Phe mutation was initially clinically indexed as CAIS, but at gonadectomy remarkable Wolffian duct development became evident, suggesting important paracrine androgen action, indicating a PAIS phenotype.

Intranuclear mobility of all mutants was studied with FRAP, a technology that can provide information on the DNA-binding potential [Van Royen et al., 2009, 2011]. All transcriptional activation inactive mutants (p.Arg609Met, p.Leu701Phe, p.Ser741Tyr, p.Arg775Leu, and p.Lys913X) displayed lack of immobilization similar to a non-DNA-binding mutant control p.Ala574Asp [Brüggenwirth et al., 1998; Farla et al., 2004; Van Royen et al., 2007]. Intermediate values for the redistribution were found for p.Leu723Phe and p.Asp691del. These mutants also displayed intermediate or low transcriptional activation activities. In that respect, the FRAP data fitted well with the results from the other functional assays.

The detailed structural modeling information was an important additional support in predicting the molecular phenotype of the mutant receptor. These predictions could only be done for mutations in the DBD and LBD, because these subdomains have been crystallized [Matias et al., 2000; Sack et al., 2001; Shaffer et al., 2004]. None of the mutations is in one of the 18 residues forming the ligand-binding pocket [Matias et al., 2000] or is part of the recently described BF3 (Binding Function 3) surface on the LBD of nuclear receptors including the AR, which can be considered as an additional docking site for coactivators and an allosteric modulator of the adjacent AF-2 pocket [Buzón et al., 2012; Grosdidier et al., 2012].

With respect to treatment and counseling of AIS individuals, it is important that in those cases where no gonadectomy has been performed, the partial activity of the mutant receptor might become a matter of concern during puberty and thereafter, in particular if these AIS patients are raised as girls. Gonadal steroidogenesis may cause a variable degree of virilization. Although it is generally recognized that gonadal tumor risk is slightly higher in PAIS than in CAIS, current knowledge does not allow to predict the risk for development of a gonadal germ cell tumor from the results of in vitro functional assays [Cools et al., 2011].

In conclusion, the present investigation on novel AR mutations in AIS patients reveals that a combination of functional assays and structural analyses is a preferred approach for assessment of the severity of the mutation and for prediction of possible 'rest-activity' of the mutant receptor. An extensive functional classification of unknown AR sequence variants, as provided in the current study, is of highly relevant importance for clinical patient management, counseling strategies and future outcome predictions.

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