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Research Article

Unfaithfulness and promiscuity of a mutant androgen receptor in a hormone-refractory prostate cancer

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Abstract. Missense mutations in the androgen receptor (AR) contribute to the failure of hormonal therapy for prostate cancer (PCa), but the underlying molecular bases remain uncharacterized. Here, we describe a new AR variant found in a hormone-refractory metastatic PCa, in which threonine 575 in the DNA binding domain, and threonine 877 in the ligand-binding domain, were both replaced by an alanine. Using gene reporter assays, we demonstrate that the T575A mutation weakened transcriptional activity from promoters containing AR-specific responsive elements, while activity from promoters with AR-non-specific elements was enhanced. Data from gel shift experiments revealed a preferential binding of the T575A mutant to AR-non-specific motifs. We demonstrate that the two mutations T575A and T877A cooperate to confer new functional properties on the AR, and that the mutant AR functions simultaneously as a promiscuous AR due to the T877A mutation, and an unfaithful AR due to the T575A mutation.

Key words. Androgen receptor; hormone-refractory prostate cancer; mutation; DNA binding specificity; transcriptional activity.

The biological actions of the male sexual hormone dihydrotestosterone (DHT) are mediated by the androgen receptor (AR), a member of the steroid-thyroid-retinoid-vitamin D superfamily of nuclear receptors, which function as ligand-activated transcription factors [1]. Like other members of the nuclear receptor family, the AR is structured in different domains and motifs reflecting its activation and function through ligand binding, phosphorylation, nuclear translocation, dimerization, DNA binding to cognate androgen response elements (AREs) present in the promoter of target genes, cofactor recruitment by protein-protein interactions, and transcriptional activation [2].

The AR modulates the rate of transcriptional initiation through association with the basal transcription machinery and alterations in the state of chromatin organization at the promoter of target genes. This is achieved by a specific recruitment of members of the p160 family of histone acetyltransferase coactivators including steroid receptor coactivator (SRC)-1, SRC-2, SRC-3, p300/CREB-binding protein (CBP), and pCAF [3], or by the recruitment of corepressors like nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT) with their associated histone deacetylases [4]. This genotropic pathway is well documented for the control of the expression of the human *prostate-specific antigen (hPSA)* gene, a target of the AR

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in prostate tissue [5]. The *hPSA* promoter contains three putative AREs, AREI (–170 pb) and AREII (–394 bp) within the proximal region, and AREIII (–4.2 kb) in the distal enhancer region [5, 6]. In this model, agonist-bound ARs are recruited to both the proximal promoter (AREII) and the enhancer element (AREIII), followed by a coordinated and ordered recruitment of p160 proteins, p300/CBP and RNA polymerase II holoenzyme. On the other hand, the formation of an antagonist-bound AR corepressor complex involves only AREI in the proximal promoter.

As the three androgen-responsive motifs in the *hPSA* promoter differ in their DNA sequence [6], an intriguing question is how the selectivity of AR DNA binding is achieved. This study of the formation of the AR transcription complex on the *hPSA* promoter and previous data suggest that the DNA binding should be determined by the type of ligand bound to the AR, but also by its interaction with either coactivators or corepressors [5–7].

Similarly, the way the specificity of DHT action mediated by the AR is obtained remains unclear. Indeed, the class I subgroup of the nuclear receptor (NR) superfamily, including the AR, the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR), and the progesterone receptor (PR), can recognize the same three-nucleotide spaced inverted repeats (IR3) of the 5′-TGTTCT-3′-like monomer-binding motif present in the promoter of target genes [8]. The relative balance of receptors, coactivators, and corepressors has been suggested to be a critical determinant to initiate a specific response. Additionally, recent studies suggest that the specificity of the androgen signalling pathway relies on an alternative DNA-dependent dimerization of the AR on direct repeats of the monomer-binding motif rather than inverted repeats, and an active role of sequences flanking these response elements [9]. However, our understanding of how the AR selectively regulates the expression of genes at the transcriptional level is still incomplete.

Androgens are involved in prostate cancer (PCa) cell growth and survival, and androgen ablation therapy remains the mainstay of treatment for patients with advanced PCa. Despite the initial repressive effects, all PCas will switch from an androgen-dependent to an androgen-independent state in which the AR continues to play a crucial role by allowing tumour cell growth in an androgen-depleted environment [10, 11].

AR mutations are one of the proposed mechanisms to explain how PCa can escape androgen deprivation [12]. To date, 85 AR mutations have been found in PCas, almost all being single-base substitutions due to somatic mutations [13]. Nearly 45% of these mutations occur in the ligand-binding domain (LBD) of the receptor, where they can modify the sensitivity of the AR for steroid hormones other than androgens, or for non-steroid molecules such as antiandrogens [14, 15]. Mutations in the LBD by affecting the ligand-induced AR conformation might also decrease the recruitment of corepressors [16]. These naturally occurring mutations in the AR undoubtedly provide precious information for a better understanding of AR-specific transcriptional activation.

In the present study, we describe a novel AR variant carrying the T575A/T877A double mutation, isolated from a hormone-refractory metastatic PCa.

The T877A mutation is currently described in the literature. This mutation is known to lead to promiscuous ligand activation of the AR. Indeed, the replacement of the threonine in position 877 by an alanine enlarges the ligand-binding pocket allowing the binding of the antiandrogen, hydroxyflutamide, as an agonist. Similarly, the binding of steroid molecules with a larger substitution at carbon 17 in the D ring, like cortisol and 17α-hydroxyprogesterone (17α-OH-Prog) is favoured [14, 15]. More recently, the T877A mutation was discovered to affect the ligand-induced conformational change of the AR, and to considerably reduce the repressive action of the corepressor NCoR. This lack of NCoR action would allow antiandrogens to act as strong agonists [16].

The single T575A AR mutation has already been reported in another metastatic PCa but the functional consequences of this mutation on AR activity have not been investigated so far [17]. The T575A mutation is localized in the first zinc finger of the DNA-binding domain (DBD), just before the P-box, which intervenes in ARE recognition.

To define the consequences of the T575A mutation on AR functions, a comprehensive analysis of AR transcriptional activities from different hormone-responsive promoters was conducted. The present study found that the T575A mutation decreased AR transcriptional activities from promoters containing AR-specific hormone-responsive elements (HREs) and increased AR activities from promoters containing AR-non-specific HREs. Data from gel shift experiments showed that AR DNA binding to non-specific HREs was also enhanced in the presence of the T575A mutation. These findings suggest that threonine in position 575 probably orientates AR DNA binding to specific HREs. Furthermore, we demonstrate that in the T575A/T877A mutant AR, the effects of the two mutations combine, and give rise to an AR that functions simultaneously as a promiscuous AR due to the T877A mutation, and an unfaithful AR due to the replacement of threonine in position 575 by alanine.

Materials and methods

Materials. All steroids, flutamide, yeast medium components, phenol-red-free Dulbecco's modified Eagle's medium, mycoplasma-screened fetal calf serum (FCS)

were from Sigma Aldrich. Primers were from MWG Biotech.

Plasmids. The plasmids pAR_{WT} and pAR_{T877A} for expression of the wild-type (wt) AR and the T877A mutant AR in yeast have already been described [18]. The plasmid $pAR_{T575A/T877A}$ was rescued from yeast clones expressing the T575A/T877A double-mutant AR as previously described [19]. The pAR_{T575A} single mutant AR was obtained from $pAR_{T575A/T877A}$ by exchanging the *Eco*RI–*Eco*RI fragment (NM_000044: nt 3521–4015) with the wt counterpart. For cell transfections, the expression vector for the full-length human AR ($pSV\text{-}AR_0$), a gift from Dr. A. O. Brinkmann (Erasmus University, Rotterdam), was used. To generate $pSV\text{-}AR_{MUT}$ constructs, the following forward 5′-TGCGGCGGCGCAGTGCCGCTAT-3′ (NM_000044: nt 2311–2332) and reverse 5′-TAGGGATCCAATGCTTCACTGGG-3′ (NM_000044: nt 3867–3890) primers were used to amplify a *Bst*EII–*Bam*HI fragment from pAR_{T575A}, pAR_{T877A}, and $pAR_{T575A/T877A}$, which was thereafter exchanged with the corresponding fragment in $pSV\text{-}AR_0$, yielding $pSV\text{-}AR_{T575A}$, $pSV\text{-}AR_{T877A}$, and $pSV\text{-}AR_{T575A/T877A}$, respectively. The underlined sequence in the reverse primer refers to the *Bam*H I site. The GREcs plasmid was constructed by inserting the consensus glucocorticoid responsive element (GRE) sequence, 5′-TGTACAggaTGTTCT-3′, in the multiple cloning site of the pTAL-Luc plasmid (BD Clontech) between *Mlu*I and *Eco*RI restriction sites. All constructs were checked by sequencing. The MMTV-Luc reporter plasmid containing GREs was a gift from Prof. P. Chambon (IGBMC, Illkirch, France). The PSA-61-Luc reporter plasmid containing a 6-kb *PSA* promoter fragment with the three AREs (AREI, AREII, and AREIII) was from Dr. Trapman (Erasmus University, Rotterdam). *sc*-ARE1.2, *slp*-HRE2, and C3(1) ARE luciferase reporter plasmids were from Dr. F. Claessens and Dr. G. Verrijdt (Faculty of Medicine, University of Leuven, Belgium). pEGFP-C3 was purchased from BD Clontech.

Tissue acquisition. The patient was diagnosed with T3NxMo PCa and was first treated with leuprorelin and flutamide. The patient responded to this complete androgen blockade for 17 months and relapsed thereafter. After informed consent, 3 ml of bone marrow was aspirated from the right posterior iliac crest, 5 years after the initial diagnosis. Cytologic examination showed the presence of numerous metastatic PCa cells in the bone marrow aspirate.

***ADE2* reporter assay.** The assay was performed in the EJ250 yeast strain in which the expression of the *ADE2* gene, required for adenine biosynthesis, was androgen dependent [18]. The *ADE2* reporter gene was placed under the tight control of a yeast minimal promoter linked to three repeats of the sequence 5′-AGAACAgcaAGTGCT-3′, corresponding to AREI (–170 bp) of the human *PSA* gene. Yeast cells were transformed by the LiAc/PEG method with 1 µg expression plasmid encoding either wt AR or the indicated mutant AR. Transformed yeast cells were then plated on selective medium depleted in adenine and containing increasing concentrations of a panel of steroid or non-steroid ligands, or the vehicle ethanol as the negative control. Colonies obtained on each plate were scored, reflecting the transcriptional activity of the AR in response to the ligand tested. In the positive control (+ ade), transformed yeast were plated on selective medium, which did not contain any ligand, but was supplemented with 200 µg/ml adenine. In such conditions, the androgen-dependent expression of the *ADE2* gene was not required for yeast growth.

Cell lines. The monkey kidney cell lines CV-1 and COS-1, obtained from Prof. P. Chambon (IGBMC, Illkirch, France), were grown in Dulbecco's modified Eagle's medium supplied with 10% FCS, 2 mM glutamine, 10 U/ml penicillin, and 10 U/ml streptomycin (all Sigma-Aldrich).

Luciferase reporter assays. Transient transfections were performed in CV-1 cells using a JetPEI reagent (PolyPlus Transfection) according to the instructions provided by the manufacturer. For luciferase assays using pMMTV-Luc or PSA-61-Luc as reporter plasmids, CV-1 cells were seeded into 12-well plates, and transfected with 1 µg of AR expression constructs in combination with 2 µg of reporter plasmid and 50 ng of pEGFP-C3 as the internal control. For luciferase assays using *sc*-ARE1.2, *slp*-HRE2, or C3(1)-ARE as reporter plasmids, CV-1 cells were into 24-well plates, and transfected with 200 ng of AR expression constructs in combination with 400 ng of the indicated reporter plasmid and 200 ng of pEGFP-C3 as the internal control. All transfections were performed in phenol-red-free medium containing 10% dextran-coated charcoal-stripped FCS, DHT or 17-α-OH-Prog at the indicated concentration, or vehicle (ethanol). Forty-eight hours after transfection, cell lysates were harvested and analysed for firefly luciferase activity with reagents from the Luciferase Reporter Assay System (Promega) using the TD-20/20 luminometer (Turner Designs).

Preparation of cell extracts. COS-1 cells were plated at 2×10^6 cells/10-cm dish and transfected with 15 µg of AR expression plasmid and JetPEI reagent (PolyPlus Transfection), and incubated in phenol-red-free medium containing 10% dextran-coated charcoal-stripped FCS and 100 nM DHT for 48 h. Cells were then washed twice with ice-cold 1×PBS containing 1× protease inhibitor cocktail from Sigma, scraped into 1 ml of chilled PBS with 1× protease inhibitor cocktail, and were pelleted by centrifu-

gation at 1700 g for 5 min at 4 °C. Cell pellets were resuspended in 400 µl of ice-cold buffer A [10 mM Hepes KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulphonyl fluoride (PMSF), and 1× protease inhibitor cocktail] by gently flicking the tube, and left on ice for 10 min. Cell lysates were vortexed briefly, and centrifuged at 10,000 g for 30 s. Pellets were resuspended in 50 µl of ice-cold buffer C (20 mM Hepes KOH pH 7.9, 420 mM KCl, 1.5 mM $MgCl_2$, 25% glycerol, 0.5 mM dithiothreitol, 0.2 mM PMSF, and 1× protease inhibitor cocktail), and incubated on ice for 30 min. Cell debris was removed by centrifugation at 10,000 g for 2 min at 4 °C, and supernatants were stored at – 80 °C.

Electrophoretic gel mobility assay. Electrophoretical gel mobility assays **(**EMSAs) were performed essentially according to De Vos et al. [20]. Oligonucleotides (table 1) were hybridized and ^{32}P-labelled in a fill-in reaction carried out in 20 µl of buffer containing 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 7.5 mM dithiothreitol, 0.15 mM of each dTTP, dATP and dGTP, 0.2 µM of double-stranded oligonucleotides, 1 µCi/ µl of [α-^{32}P]dCTP (Amersham Pharmacia Biotech), and 0.25 U/ µl of the Klenow fragment of DNA polymerase I (BioLabs) for 30 min at 37 °C. The reaction was then stopped by adding EDTA to a final concentration of 10 mM, and unincorporated [α-^{32}P]dCTP was removed from the labelled probes by purification on MicroSpin G-25 Columns (Amersham Biosciences). Constant amounts (120,000 cpm) of labelled probes were incubated for 15 min on ice with nuclear extracts from transfected COS-1 cells, in 40 µl of binding buffer [20 mM Hepes KOH pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol, 100 mM NaCl, 0.05% Triton X-100, 50 ng poly(dI-dC), and 1 mM dithiothreitol]. Subsequently, free or bound probes were separated by electrophoresis on a 5% (w/v) acrylamide gel (acrylamide/bisacrylamide, 29:1, w/w) containing 0.25× Tris/borate/EDTA, and 0.05% Triton X 100 (Sigma-Aldrich) for 5 h at 120 V, and at room temperature. The percentage of retarded probe was determined by scanning dried gels with a PhosphoImager (Molecular Dynamics) and by densitometric studies using the Quantity One software (Bio-Rad).

Table 1. Comparision of AR-non-specific and AR-specific HREs.

Name	Sequence	Specificity
C3(1) ARE	5′-AGTACGtgaTGTTCT-3′	non-specific
GRE consensus	5′-TGTACAggaTGTTCT-3′	non-specific
sc-ARE1.2	5′-GGCTCTttcAGTTCT-3′	AR specific
slp-HRE2	5′-TGGTCAgccAGTTCT-3′	AR specific
PSA-AREI	5′-AGAACAgcaAGTGCT-3′	AR specific
ARE consensus	5′-GGTACAgggTGTTCT-3′	AR specific
	–7 0 7	

Numbering is relative to the central nucleotide of the three-nucleotide spacer.

Immunoblotting. Protein extracts were prepared from yeast cells as described previously [18]. Equal amounts of protein were loaded on a 7.5% SDS PAGE and transferred onto nitrocellulose membranes by electroblotting. Blots were probed with the rabbit polyclonal IgG antibody sc-13062 against human AR (Santa Cruz Biotechnology) and peroxidase-conjugated secondary antibody (goat anti-rabbit PO; Bio-Rad). Detection was carried out with the chemiluminescence Western blotting kit (Amersham Biosciences).

Homology modelling. The *in silico* modelling of DNA-bound wt AR DBD was realized based on the structure of the human GR DBD (pdb entry 1GLU) [21], using the Modeller software and default parameters [22], the Turbo-Frodo software, and the lsq-man option in the O package software. The quality of the models was evaluated with both Procheck [23] and ProsaII [24], and was compared with the recently published structure of the AR-DBD2-DNA crystal (pdb entry 1R4I) [25].

Results

Transcriptional activities of a new AR variant with the T575A/T877A double mutation isolated from a hormone-refractory PCa. We have developed a yeast functional assay to screen PCa samples for AR mutations, and to analyse the transcriptional activities of the mutant ARs in the presence of a panel of agonists or antagonists [18]. By applying this assay to study AR status in a hormone-refractory metastatic PCa as previously described [19], we detected a novel mutant AR variant carrying the T575A and T877A substitutions. This is the first description of this T575A/T877A AR variant. The T877A mutant AR has been described in hormone-refractory prostate cancer, but little is known about the effect of the T575A mutation on AR activities, or about the consequences of the two mutations within the same AR molecule.

We first evaluated transcriptional activities of this T575A/T877A AR variant in our yeast functional assay. As controls, the wt AR and the AR variant carrying the T575A or the T877A mutation alone were also assayed. While wt AR transcriptional activity in the presence of low concentrations of DHT (0.01–0.1 µM), and higher doses (1–10 µM) of androstenedione, β-estradiol, progesterone, medroxyprogesterone, and 17α-OH-Prog was strong as expected (fig. 1A) [18, 19], no transcriptional activity was observed with the T575A/T877A mutant AR, whatever the ligand added into the medium, and the concentrations tested (fig. 1B). Yeast colonies were only observed on the

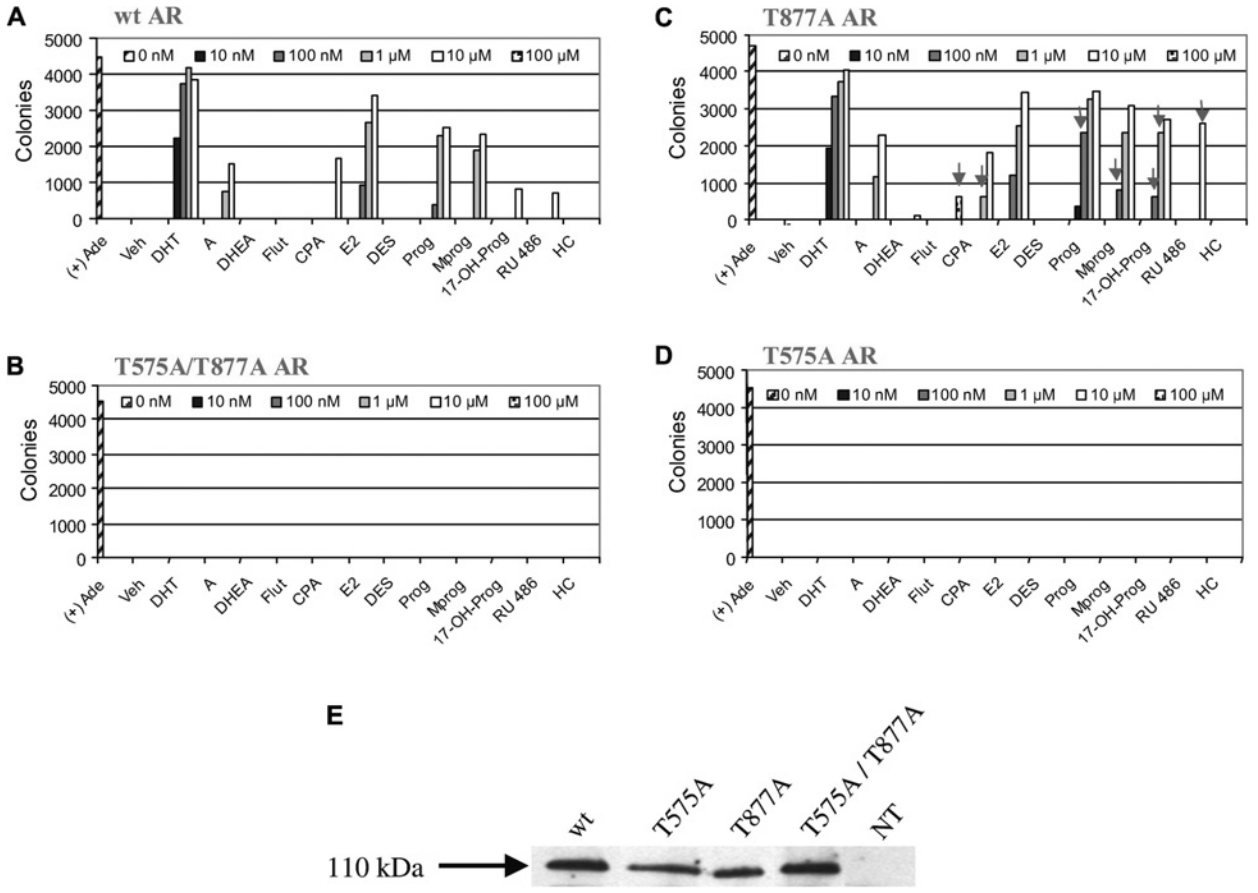


Figure 1. Activation of transcription from an ADE2 gene reporter assay by the wt and mutants ARs. Yeast cells were transformed as described in Material and methods with the expression plasmid encoding the wt AR (*A*), the T575A/T877A (*B*), the T877A (*C*) or the T575A (*D*) mutant ARs, and were plated on selective media containing one of the following steroid or non-steroid ligands: DHT, dihydrotestosterone; A, androstenedione; DHEA; Flut, flutamide; CPA, cyproterone acetate; E2, β-estradiol; DES, diethylstilbestrol; Prog, progesterone; Mprog, medroxyprogesterone; 17-OH-Prog, 17α-OH-progesterone; RU486, mifepristone; HC, cortisol. Histograms represent the number of colonies obtained with each concentration of ligand (10 nM, 100 nM, 1 µM, 10 µM). Flut was tested at 0.1–100 µM. Data represent one experiment performed three times. Mutation-induced changes in hormone response are depicted by arrow. (*E*) Western blot analysis of AR expression in yeast cells. Protein extracts from yeast cells expressing either the wt AR, the T877A, the T575A/T877A, the T575A mutant ARs or from non-transfected yeast cells were separated on 7.5% SDS PAGE. Blots were revealed with the rabbit polyclonal IgG sc-13062 against AR (110 kDa).

positive control plate (fig. 1B, + Ade), which was supplemented with adenine. In these conditions, AR transcriptional activity was not required for yeast growth.

The impaired transcriptional activity observed with the T575A/T877A mutant AR cannot be attributed to the sole T877A mutation in the LBD as this mutation is known, on the contrary, to broaden the response of the AR to antiandrogens and to several other steroid hormones. Indeed, AR transcriptional activity in the presence of the T877A mutation alone was observed, as expected, from a lower concentration (100 nM) of progesterone, medroxyprogesterone, and 17α-OH-Prog compared with the wt AR (fig. 1C) [19]. The T877A mutant AR also responded to the full antagonist flutamide, and to the partial agonist CPA and RU486.

We next tested whether the T575A mutation alone in the DBD might explain the impaired transcriptional activities observed with the T575A/T877A mutant AR. The AR with the single T575A mutation was created by mutagenesis from the wt AR and assayed in the yeast system. As expected, the T575A mutant AR was unable to up-regulate the expression of the *ADE2* reporter gene in response to hormone stimulation in our yeast model (fig. 1D).

Western immunoblotting was performed to exclude the possibility that the absence of transcriptional activities observed with the T575A and T575A/T877A mutant ARs was due to a decrease in AR protein levels. As shown in Figure 1E, no AR expression was observed in non-transfected yeast cells, while the expression of the T575A and T575A/T877A mutant ARs in yeast cells was comparable with that of the wt or T877A mutant AR. Collectively, these results indicate that the T575A mutation within the AR DBD should impair AR transcriptional activities from our *ADE2* gene reporter construct. The presence of the T575 mutation should affect somehow the recognition of the responsive element that has been inserted in this artificial

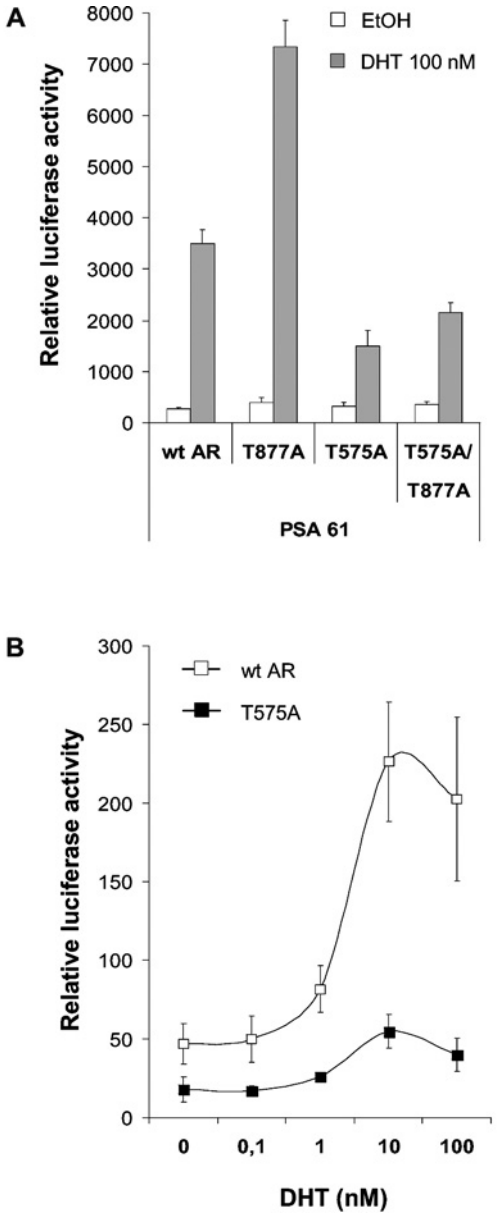


Figure 2. The T575A mutation attenuates AR transcriptional activity from the human *PSA* promoter. CV-1 cells were cotransfected with expression vectors for wt or mutant AR and PSA-61-Luc. Transfected cells were treated with either DHT, or vehicle (ethanol) in steroid-depleted medium for 48 h. Cell extracts were subsequently assayed for luciferase activity. (*A*) Transcriptional activities of the wt, the T877A, the T575A, and the T575A/T877A mutant ARs in response to 100 nM DHT. (*B*) Dose-dependent response of wt and T575A mutant ARs to DHT stimulation. Values are given as arbitrary units. Data represent the mean of assays performed in triplicate ± SE.

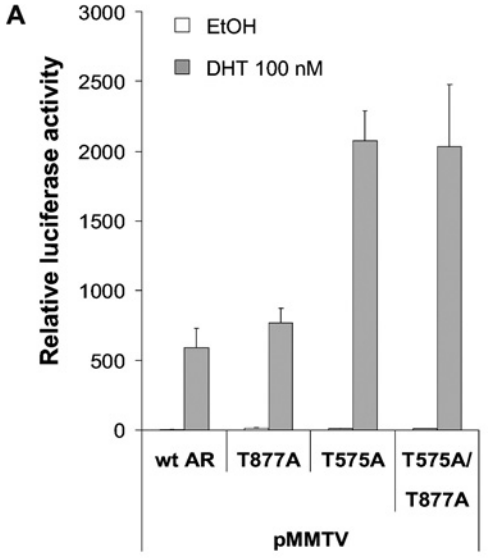
promoter, and that corresponded to AREI (–170 pb) of the *hPSA* gene. To test this hypothesis, a comprehensive analysis of different hormone-responsive promoter constructs, in which the luciferase reporter gene was placed under the control of identified steroid HREs, was performed in CV-1 cells.

Effect of the T575A mutation on AR transcriptional activities in CV-1 cells. The transcriptional activities of the wt AR, the T575A, T877A, and T575A/T877A mutant ARs were first evaluated on the PSA-61-luciferase construct. This complex promoter corresponds to a 6-kb-long fragment of the human *PSA* gene, and contains three AREs, AREI (–170 pb), AREII (–394 pb), and AREIII (–4.2 kb) [6]. Cotransfected CV-1 cells were incubated in the presence of either 100 nM DHT or vehicle. The wt AR and the T877A AR led to a strong up-regulation of luciferase activity after DHT treatment (10- and 22-fold, respectively) (fig. 2A). As expected, both the T575A and the T575A/T877A mutant ARs led to a weak induction of luciferase activity (4.5- and 6.4-fold. respectively) from this *PSA* gene promoter. Dose response curves were also performed in CV-1 cells to confirm that the weak response of the AR with the T575A mutation from the PSA-61 promoter was independent of the concentration of DHT (fig. 2B). These data supported the results obtained in our yeast-based reporter system, in which ARs with the T575A mutation were less responsive.

To investigate whether the T575A mutation affects the recognition of a different complex promoter, we next tested the MMTV-LTR-luciferase reporter construct, which contains GREs instead of AREs. The wt AR like the other members of class I nuclear receptors such as the GR, the MR, and the PR is known to be transcriptionally active from this promoter. The T575A mutant AR led to an unexpected strong up-regulation of luciferase activity after DHT stimulation, compared with the wt AR (244- and 70-fold, respectively) (fig. 3A). Similarly, the T575A/T877A mutant AR had a strong activity from the mouse mammary tumour virus (MMTV) promoter after DHT stimulation compared with the T877A mutant AR (240- and 91-fold, respectively). Similar results were obtained with the simple GRE-TATA-luciferase reporter construct. The T575A mutant AR activity in the presence of DHT was strongly increased when compared with the wt AR and the T877A mutant AR (fig. 3B).

We next extended the analysis of T575A mutant AR transcriptional activities to two other AR-specific AREs, the *slp*-HRE2 from the mouse *sex-limited protein* (Slp) gene and the *sc*-ARE1.2 from the *human secretory component* (SC) gene, and to another AR-non-specific HRE, the C3(1)-ARE from the rat C3(1) gene [8, 20]. Compared with the wt AR, the T575A mutant AR was, as expected, less active on the *sc*-ARE1.2 and the *slp*-HRE2 promoter (fig. 4A), and more active upon the C3(1)-ARE promoter (fig. 4B).

Together, these results indicate that the T575A mutation impairs AR transcriptional activities from promoter constructs containing AR-specific HREs, but increases AR activities from promoters containing AR-non-specific HREs.



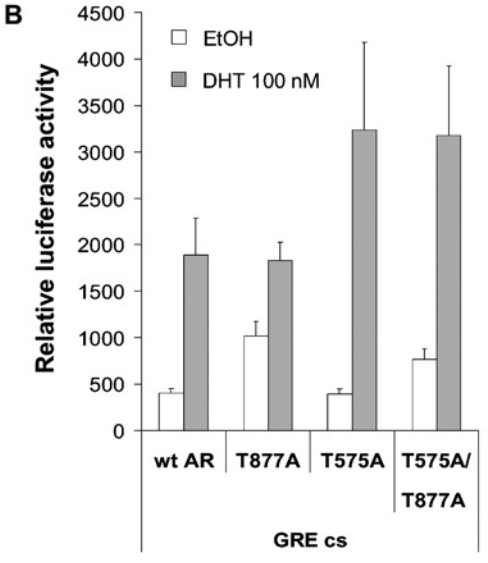
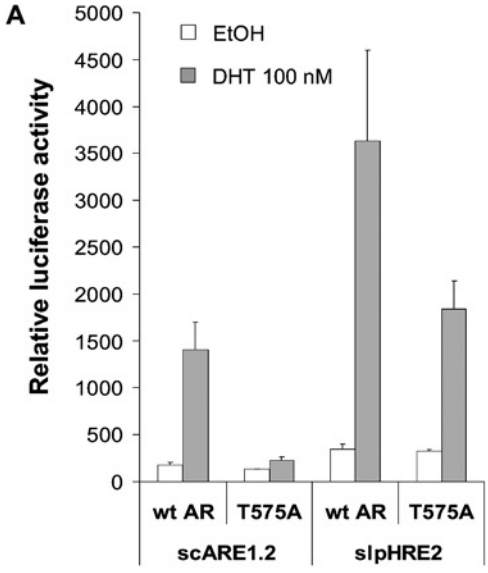


Figure 3. The T575A mutation increases AR transcriptional activity from the MMTV-Luc and GRE-Luc promoter constructs. CV-1 cells were cotransfected with expression vectors for wt or mutant AR and MMTV-Luc (*A*) or GRE-Luc (*B*) promoter constructs. Transfected cells were treated with either 100 nM DHT or vehicle in steroid-depleted medium for 48 h. Cell extracts were subsequently assayed for luciferase activity. Values are given as arbitrary units. Data represent the mean of assays performed in triplicate ± SE.



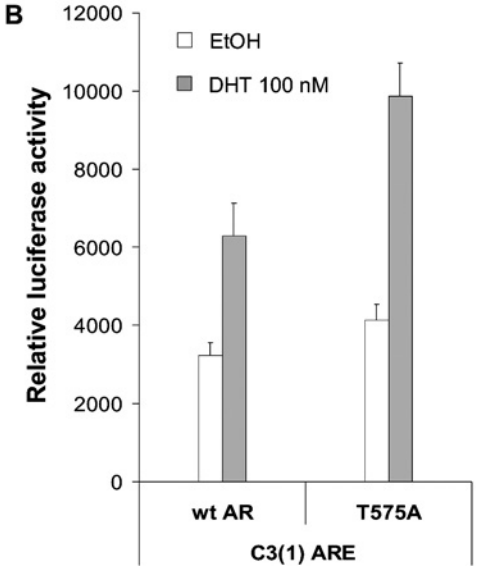


Figure 4. The T575A mutation affects the specific recognition of HREs by the AR. CV-1 cells were transfected with expression vectors for wt or mutant AR and the high-affinity and AR-non-specific C3(1)-ARE-Luc (*A*), or the high-affinity and AR-specific *sc*-ARE1.2 and *slp*-HRE2 (*B*), as well as pEGFP-C3. Cells were treated with 100 nM DHT in steroid-depleted medium for 48 h. Cell extracts were subsequently assayed for luciferase activity. Values are given as arbitrary units. Data represent the mean of assays performed in triplicate ± SE.

Effects of the T575A mutation on AR DNA binding. An EMSA was further performed in COS-1 cells to investigate whether the T575A mutation affects AR DNA binding. The oligonucleotides used corresponded to two AR-specific AREs, the AREI of the *hPSA* gene (AREI-PSA), and the consensus ARE (AREcs), and to two AR-non-specific HREs, the C3(1)-ARE, and the consensus GRE (GREcs) (table 1) [9]. Nuclear extracts from transfected COS-1 cells expressing either the wt AR or the T575A AR were incubated with ^{32}P-labelled oligonucleotides, and AR-DNA complexes were resolved. The binding of T575A mutant AR to the AREI-PSA and to AREcs was significantly lower compared to that of the wt AR (fig. 5). On the other hand, the T575A mutation

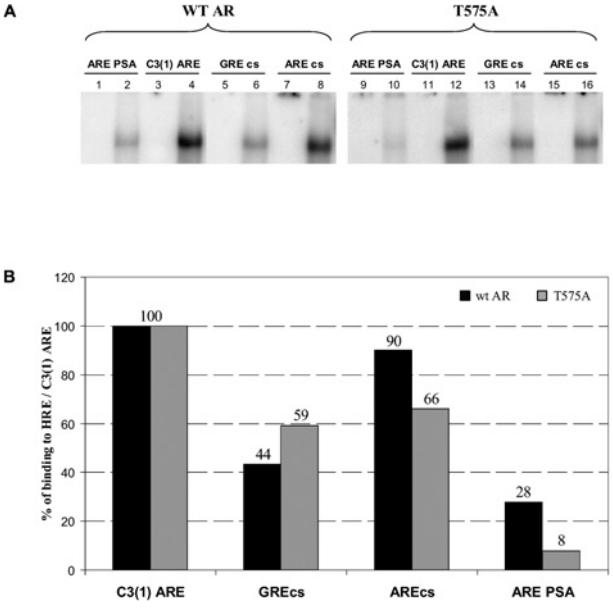


Figure 5. DNA binding activity of the wt and mutant ARs as determined by EMSA. Nuclear extracts from COS-1 cells transfected with the expression vectors for wt and mutant ARs were prepared as described in Materials and methods. (*A*) Aliquot of each sample (30 µg protein) incubated with the indicated ^{32}P-labelled HRE (even numbers), or free probes (odd numbers) were loaded on a 5% polyacrylamide gel. Identical results were obtained in repeated experiments. (*B*) Comparative analysis of AR affinity for HREs. wt and mutant ARs binding to the C3(1)-ARE was set at 100%.

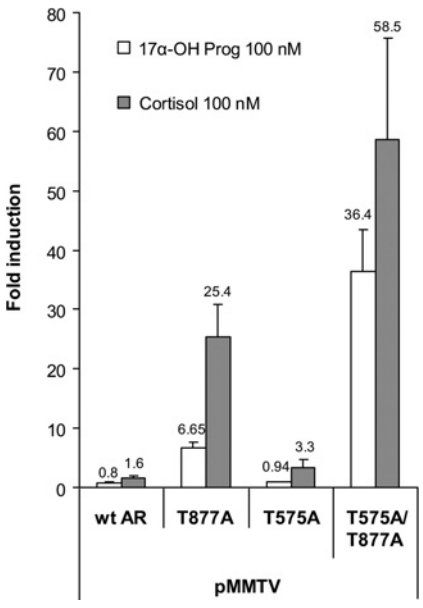


Figure 6. The T575A/T877A mutant AR is an unfaithful and promiscuous AR. CV-1 cells were cotransfected with expression vectors for wt or mutant AR and the MMTV-Luc as well as pEGFP-C3. Cells were treated with 100 nM 17α-OH-Prog or 100 nM cortisol in steroid-depleted medium for 48 h. Cell extracts were subsequently assayed for luciferase activity. Values are given as fold induction over activity found in control cells treated with ethanol. Data represent the mean of assays performed in triplicate ± SE.

enhanced AR DNA binding to the non-specific GREcs and C3(1)-ARE. Similar data were obtained with the T575A/T877A mutant AR (data not shown). These data show collectively that the T575A mutant AR displays altered DNA binding to an AR-specific ARE, but a higher DNA binding to the AR-non-canonical HREs, reflecting the transcriptional activities observed with this mutant AR.

Synergetic effects of the double T575A/T877A mutation on AR transcriptional activities. We have demonstrated that the T575A mutant AR exhibits higher DNA binding to AR-non-specific HREs and increased transcriptional activities from promoters containing such HREs. Moreover, as previously described [15, 18] and as illustrated in figure 1C, the AR response to several endogenous steroid hormones and non-steroid molecules is markedly changed in the presence of the T877A mutation in the LBD. We next tested the combined effects of the two T575A and T877A mutations on AR activity. CV-1 cells were cotransfected with either wt, T575A, T877A, or T575A/T877A mutant AR together with the MMTV-luciferase reporter plasmid and were incubated in the presence of 17α-OH-Prog or cortisol. The wt and T575A ARs, which do not contain any mutation in the LBD, remained unresponsive to 17α-OH-Prog or cortisol stimulation. As expected, transcriptional activities of the AR T877A mutant after 17α-OH-Prog or cortisol stimulation were increased (6.7- and 25.4-fold, respectively). In the T575A/T877A mutant AR, the effects of the two mutations combined to give rise to a receptor with stronger transcriptional activities from the MMTV promoter (fig. 6). Together these results indicate that the two mutations T575A and T877A cooperate to confer new properties on the T575A/T877A mutant AR: the T877A mutation by enlarging the sensitivity to hormones other than androgens, and the T575A mutation by modifying the affinity of the AR for HREs.

Discussion

We report here the characterization of a T575A/T877A mutant AR detected in a hormone-refractory metastatic PCa. An AR variant with the single T575A mutation has already been described in a metastatic PCa, but the functional consequences of this mutation on AR activity have not been investigated so far [17]. In this report we have evaluated the molecular consequences of the T575A/T877A double mutation on AR activity. We showed that the T575A mutation affects AR transcriptional activities upon hormonal stimulation in a promoter-dependent manner. In an *ADE2* reporter assay performed in yeast, in which the reporter gene was placed downstream of a min-

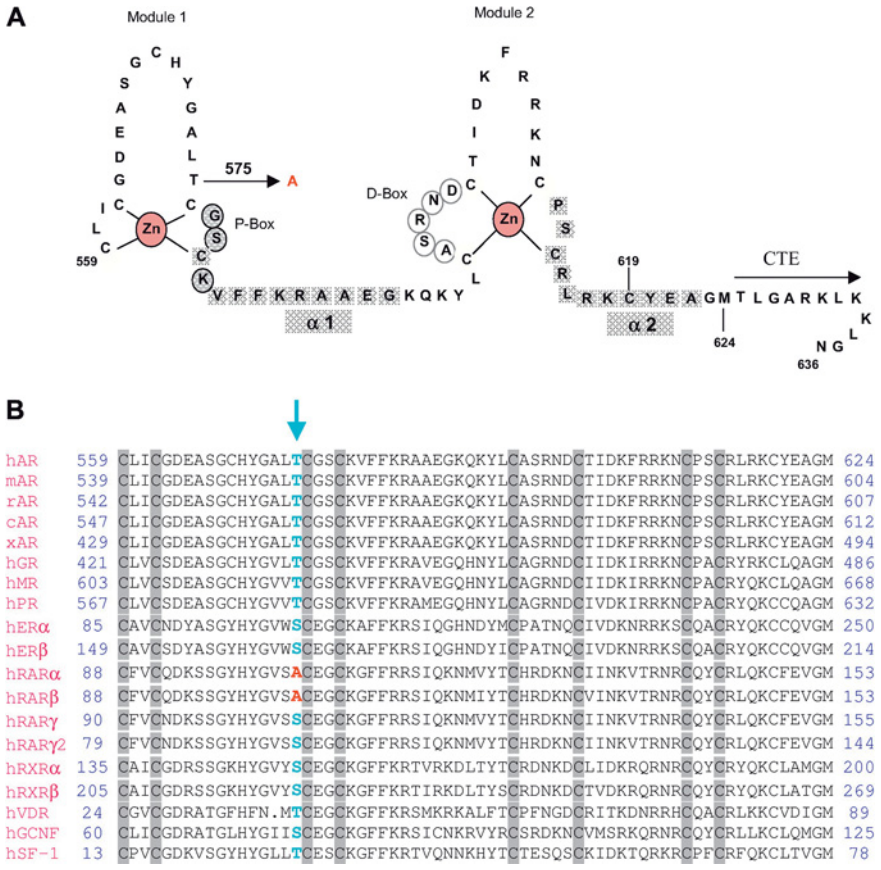


Figure 7. The hydroxyl group in position 575 in the AR DBD is well conserved among the NR family. (*A*) Schematic view of the human AR DBD with the T575A mutation. The T→A mutation is located at position 575 in the first zinc module, near the P-box (circled amino acid residues). The hatched amino acid residues indicate the two helices α1 and α2. The D-box, which is involved in dimerization is indicated by open circles. The C-terminal extension (CTE) contains amino acids that are required for high-affinity binding of the AR to AREs. (*B*) Sequence comparison of NR DBDs. The sequence alignment of the DBD of different NRs was performed using the Clustal program. The grey boxes indicate the zinc-coordinating cysteines. Position T575 in the AR is indicated by the arrow. Note the high conservation of a hydroxyl group from threonine or serine in this position. The organism abbreviations are: h, *Homo sapiens*; m, *Mus musculus*; r, *Rattus norvegicus*; c, *Canis familiaris;* x, *Xenopus laevis*.

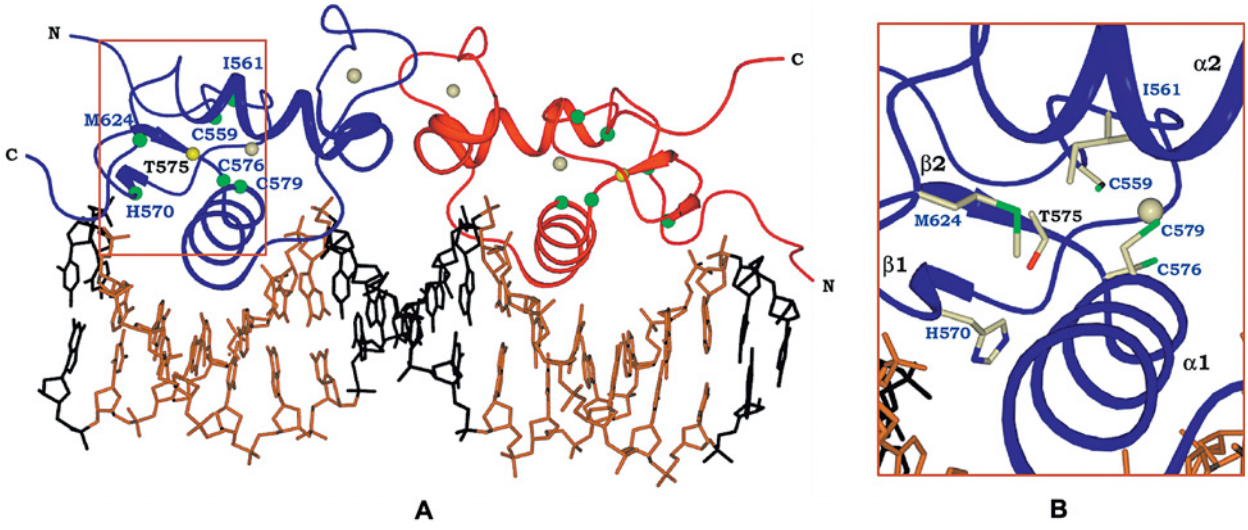


Figure 8. The human wt AR DBD-ADR3 complex model. (*A*) We performed this in *silico* modelling of the DNA-bound wt AR DBD based on the structure of the human GR DBD (pdb entry 1GLU). The two monomers are in blue and in red, the DNA half site is in brown and zinc atoms are colored in ivory. The protein is depicted as a backbone Ca trace. T575 is represented in yellow. Residues participating in the interactions with T575 are labelled in green. (*B*) Magnification of box in (*A*).

imal promoter construct with three repeats of the *cis*-acting element AGAACAgcaAGTGCT (AREI of *hPSA*, position –170), both the T575A and T575A/T877A mutant ARs were transcriptionally inactive in response to the different ligands studied. In luciferase assays performed in CV-1 cells, both the T575A and T575A/T877A mutant ARs were transcriptionally active on promoter constructs containing AR non-canonical HREs such as MMTV-Luc, GRE-Luc, and C3(1)-Luc, but were less active on promoters containing AR-specific HREs like *sc*-ARE1.2 and *slp*-HRE2. These differences in AR transcriptional activities could be explained by a weakened AR binding to specific HREs in the presence of the T575A mutation, while binding to non-canonical HREs was enhanced compared to the wt AR.

Thus, the T575A mutant AR raises the question of how AR specificity of action is obtained. The AR DBD may be considered as two interdependent modules that differ both structurally and functionally, and an amphipathic helix (fig. 7A). The helix of the first module (H1) is mainly involved in site-specific recognition based on its interaction with some bases in the cognate HRE [26, 27]. Also, within this helix are the amino acids that are responsible for site-specific discrimination of binding. These three to four amino acids have been termed the P-box. A loop formed in the second module provides the DBD homodimerization interface, and the helical region (H2) is involved in less-specific DNA interactions [28]. The T575A mutation is present in the first zinc finger module (fig. 7A), just upstream from the P-box.

It is clear that nucleotide determinants within the HRE play an important role [26, 27], like the amino acids within the P-box (fig. 7A). However, our data suggest that other amino acids within this first zinc finger module could also be important for AR-specific DNA binding. Moreover, the recent observation that position 437 within the GR, outside the P-box, also plays an important role in GR-specific DNA binding supports our data [29].

NR DBD sequence alignments (fig. 7B) show that T575 is remarkably conserved within the AR subfamily. T575 is also highly conserved among the NR superfamily, with the exception of RARα and RARβ, which exhibit an alanine residue in this position. The high degree of conservation of the hydroxyl group in this position suggests that the corresponding amino acid should be involved somehow in HRE recognition.

How the T575A mutation could affect the binding of the mutant AR to cognate HREs is not clear. Molecular modelling analyses indicate that T575 is not located at the surface of the DBD globular structure, but is completely buried (fig. 8) [25]. Indeed, T575 cannot really interact with the DNA or with the other AR monomer. In fact, T575 may play a crucial role in the AR DBD, as numerous van der Waals contacts with neighbouring amino acids are possible. These van der Waals interactions can be established with amino acids located in different helices forming the DBD, including residues C559, I561 and H570 in the first module, and residues C576 and C579 of DNA recognition helix H1 (fig. 8B). T575 may also interact with residue M624 between H2 and the C-terminal extension. Most of these contacts may be lost in the T575A mutant AR, disturbing the normal binding of the AR to the cognate DNA.

We cannot exclude the possibility that the change of threonine in position 575 to alanine affects somehow an interaction with coactivators or corepressors, and allows the AR to bind to non-specific HREs, as the AR DBD is known to interact with several coactivators and corepressors.

A detailed understanding of how these HREs are discriminated by the wt AR and the T575A mutant AR will have significant biological relevance, since the discriminating mechanisms are likely to be applicable to the entire steroid hormone receptor family.

The replacement of the threonine in position 877 by an alanine is known to enlarge the ligand-binding pocket allowing the binding of hydroxyflutamide as an agonist, but also the binding of steroid molecules with a larger substitution at C17 in the D-ring, like cortisol and 17α-OH-Prog (figs. 1C, 6), [14, 15]. Recent studies suggest that the T877A mutation affects the ligand-induced conformational change of the AR, and considerably reduces the repressive action of the corepressor NcoR. This lack of NcoR action allows antiandrogens to act as strong agonists [16]. In the light of our present data, it is tempting to postulate that the T575A/T877A mutant AR functions simultaneously as a promiscuous and unfaithful receptor, with a greater response to glucocorticoids and hydroxyflutamide due to the T877A mutation, and higher binding capacities to some AR non-canonical HREs due to the T575A mutation. Thus, a hypothesis to be tested further is that this promiscuous and unfaithful AR, by enhancing transcription from promoters containing AR-non-specific HREs, may lead to the expression of genes which usually do not depend on the AR signalling axis.

The study of the structural and functional features of these naturally occurring AR mutations can help to clarify the molecular bases of the development of hormone-refractory PCa, as well as the molecular mechanisms of AR action.

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