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Isolation and Characterization of the Androgen Receptor Mutants with Divergent Transcriptional Activity in Response to Hydroxyflutamide

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A yeast genetic screening was developed to isolate androgen receptor (AR) mutants with divergent transactivation characteristics in response to hydroxyflutamide (HF), an active metabolite of flutamide used for prostate cancer treatment. Two mutants carrying the substitution C685Y or E708K were isolated and characterized. Substitution of C685Y for wild-type AR (wtAR) rendered the receptor supersensitive to androgenic activity from HF and female hormones such as 17β-estradiol (E_2) and progesterone (P). Similar effects were observed in the AR mutant, named T876AAR, isolated from LNCaP cells. Surprisingly, we found that C685YAR7, but not T876AAR7, could be activated by casodex (bicalutamide), a nonsteroidal pure antiandrogen, with an induction fold 3- to 5-fold times higher than that for wild type or T876AAR. By contrast, although replacement of E708K for wtAR showed little effect on dihydrotestosterone-mediated transactivation, E708KAR lost its transcriptional response from many other ligands. The effects of ligands on E708KAR could be controlled at the DNA-binding level owing to the finding of a significant decrease in the DNA-binding ability once E708KAR was bound to HF, E_2, or P. Together, these results suggest that C685YAR can be a novel tool for assaying the androgenic activity from antiandrogens, and the mechanism revealed from E708KAR could provide a possible explanation for the partial androgen insensitivity syndrome in men with a natural E708KAR mutation.

Key Words: Androgen receptor; point mutation; hydroxyflutamide; antiandrogen; yeast one-hybrid system.

Introduction

Androgen receptor (AR), which mediates the physiological actions of androgens, belongs to the nuclear hormone receptor superfamily that includes receptors for steroid hormones, such as testosterone, estrogen, and progesterone (P), as well as thyroid hormone, vitamin D_3, and retinoids *(1–3)*. AR interacts with distinct DNA sequences, called androgen response elements (AREs), in target genes and functions as a transcriptional activator or repressor depending on the cell type and promoter context *(4,5)*.

The transcriptional activity of AR is regulated by its ligands *(6)*, which could be agonists or antagonists. Testosterone and 5- dihydrotestosterone (DHT) are two natural agonists for AR *(7)*, whereas environmental *(8)* or synthetic pharmaceutical antiandrogens, such as hydroxyflutamide (HF), cyproterone acetate, and casodex (bicalutamide), have been found to suppress the activity of AR *(9)*. The ability of antiandrogens to suppress the transcriptional effects of androgens has important clinical value *(10)*. However, in prostate cancer cells or tumors, instead of being inhibitory, antiandrogens can have inappropriate, agonist effects *(11,12)*. It is believed that the agonist activity from antiandrogens is the cause of the so-called antiandrogen-withdrawal syndrome, which is frequently experienced among prostate cancer patients *(13)*. A new generation of antiandrogens, which have high affinity but no agonist activity to AR, is in high demand.

The precise mechanisms by which antiandrogens activate transcription are unknown, but two models have been proposed. The first model proposed recruitment of a unique coactivator to the transcription complex by antagonist-occupied AR. A previous study showed that the AR coactivator, ARA70, could enhance the agonist activity of HF *(14)*. Thus, the ratio of corepressor to coactivator that binds to the transcription complex determines whether the outcome is inhibitory or stimulatory *(15)*. The second model proposed that AR mutations could reduce the ligand-binding specificity and result in activation of gene transcription on binding to antiandrogens or other related steroids, such as 17β-estradiol (E_2) and P. The T876AAR mutation in LNCaP cells is a typical example for this model *(16)*.

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To determine the critical amino acids within AR that mediate the agonist activity of HF, a yeast one-hybrid system was used to screen an AR mutant library. This assay incorporated a novel strategy in which the yeast cells were treated with different HF concentrations. Using this screening system, we were able to identify AR mutants based on the phenotypes of yeast growth and have isolated AR mutants that respond to HF with opposite outcomes. Characterization of the E708KAR mutant led to discovery of the mechanism for the E708KAR mutation in relation to androgen insensitivity. Characterization of the C685YAR mutant indicates it has a different mechanism from the T876AAR mutant for activation by antiandrogens, such as HF and casodex, or the related steroids. This AR mutant could be a great tool for in vitro assays to determine the agonist activity for new antiandrogens.

Results

Yeast Mutagenesis Screening for AR Mutants with Divergent Transcription Activity in Response to HF

We have developed a genetic selection assay in the yeast *Saccharomyces cerevisiae* to screen mutations within AR that respond divergently to HF. To achieve this, we created an AR-dependent survival system in which yeast *HIS3* gene was placed under control of a chimeric ARE/GAL1 promoter. When the AR transcriptional activity was induced by HF, the *HIS3* gene could be turned on for yeast survival. Without the induction of AR and the concomitant *HIS3* expression, yeast would not survive.

Libraries of AR mutants were created by random chemical mutagenesis and transformed into a yeast tester strain. Primary screenings, representing 25,000 individual transformants, were performed. Yeast colonies, containing both AR and the reporter plasmids, were replicated to –3 SD plates (lacking tryptophan, leucine, and histidine) with HF. Since our preliminary data showed that 10 μ*M* HF was necessary to induce wild-type AR (wtAR) in yeast, replica plates were screened for colonies that failed to grow in the presence of 10 μ*M* HF, or for colonies that survived in the presence of 1 μ*M* HF. Aminotriazole (a competitive inhibitor of HIS3) at a concentration of 20 m*M* was also added to increase the stringency of the screening. The secondary screening with 1 μ*M* HF resulted in the isolation of 12 clones with only 1 clone containing full-length mutated AR as determined by Western blot analysis. This was consistent with the previous report showing that the receptor becomes constitutively active when C-terminal truncation occurs, and this type of deletion-mutation represents the majority of AR gain-of-function mutations *(17)*. For the screening with 10 μ*M* HF, we isolated more than 300 clones that lost their response to HF. These clones were further screened for normal response to DHT. Seven clones were obtained, all containing full-length mutated ARs.

DNA sequence analysis of the only full-length AR mutant with an HF-supersensitive phenotype revealed a point mutation from G to A at nucleotide 2585, which changes the amino acid cysteine to tyrosine at residue 685, and was named C685YAR. On the other hand, among the seven clones with loss-of-function phenotypes to HF, four had the same E708KAR mutation with a point mutation at residue 2654 from G to A, and resulted in an amino acid change from glutamic acid to lysine. Both E708KAR and C685YAR mutations lie inside the ligand-binding domain (LBD) of AR (Fig. 1).

In yeast growth assays, the typical phenotypes of yeast transformed with the C685YAR and E708KAR mutants showed HF-inducible and HF-repressible growth, respectively (data not shown). No yeast could grow in the absence of HF, but in the presence of 1 μ*M* HF, yeast transformed with C685YAR mutant, but not with wtAR, grew abundantly. When the HF concentration was increased to 10 μ*M*, yeast transformed with the C685YAR mutant or with wtAR could grow well, whereas the yeast transformed with E708KAR mutant could not. However, E708KAR mutant–transformed yeast could grow as well as wtAR in the presence of 5 n*M* DHT, indicating that the E708KAR mutant has a normal response to DHT but not to HF. When the AR expression plasmids were recovered and reintroduced into another clone of yeast for confirmation, the same phenotypes were observed. As expected, the β-gal activity assay for these phenotypes was consistent with the result of the growth assay (data not shown).

Effect of C685Y and E708K Mutations of AR on Ligand-Binding Affinity

Competitive ligand-binding assays were performed to determine the binding profiles for wtAR, C685YAR, E708KAR, and T876AAR mutants toward DHT, HF, E_2, P, and casodex (data not shown). All mutants examined had ligand-binding affinity to DHT similar to that of the wtAR. Consistent with previous reports *(16,18)*, T876AAR had a 4- to 8-fold higher binding affinity to HF, E_2, and P, but it had a slightly lower affinity than wtAR to casodex. This is different from C685YAR and E708KAR mutants in which mutations did not significantly change the ligand-binding affinity to all ligands tested. Thus, the divergent transcriptional activity of the C685Y and E708KAR mutants observed in the yeast growth assay was not owing to a change in ligand binding characteristics.

Divergent Transactivation Characteristics of C685YAR and E708KAR in Mammalian Cells

Transactivation activity of wtAR and AR mutants was determined by transient transfection of the expression vectors into H1299 cells followed by a reporter gene assay. As shown in Fig. 2, all the AR mutants, C685Y, E708K, and T876A, had a response to DHT similar to that of the wtAR (Fig. 2A, lanes 2–4, 8–10, 14–16, and 20–22). These

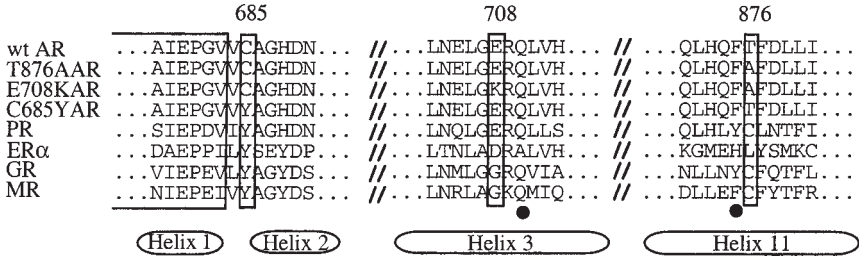

Fig. 1. Sequence alignment of local LBDs between wtAR, C685YAR, E708KAR, and T876AAR together with selected steroid receptors. Residues of mutation of ARs are boxed with the number shown on the top. Black dots indicate the amino acid residues making hydrogen bonds to bound ligands. The putative secondary structural elements are indicated below the sequences. PR, progesterone receptor; ERα, estrogen receptor α; GR, glucocorticoid receptor; MR, mineralocorticoid receptor.

mutants, however, respond differentially to other ligands; for example, the mutations at C685YAR and T876AAR rendered AR supersensitive to HF (Fig. 2A, lanes 11–13 and lanes 23–25 vs 5–7). By contrast, the mutation at E708KAR lost its response to HF even at a high concentration of HF (lanes 17–19 vs 5–7). The transcriptional activity of the C685YAR and T876AAR mutants in response to 1 μ*M* HF was 5- and 10-fold higher than that of wtAR, respectively (Fig. 2A, lanes 11 and 23 vs 5). Apparently, the T876AAR mutant could be activated at a lower concentration of HF than that for C685YAR. Peak transactivation by HF of the C685YAR and T876AAR mutants was approximately 2.5-fold higher than that of wtAR (Fig. 2A, lanes 13 and 25 vs 7). These data from mammalian cells for the ligand-regulated transactivation of AR mutants were consistent with the results obtained previously from the yeast growth assay.

As shown in Fig. 2B, the transcriptional activity of the C685YAR and T876AAR mutants could also be induced by E_2 at a concentration between 10 and 100 n*M* (lanes 12 to 13 and 24 to 25) and by P at 100 n*M* (lanes 10 and 21 to 22), respectively, as compared to wtAR. Peak transactivation by E_2 of C685YAR and T876AAR was two- and threefold higher than wtAR, respectively. These data indicated that the C685YAR or T876AAR mutation rendered AR more sensitive to the agonist activity from HF, E_2, and P. The sensitivity of AR to agonist activities from HF, E_2, and P was completely abolished when the E708KAR mutation was introduced to AR (Fig. 2A, lanes 17–19; Fig. 2B, lanes 14–19).

C685YAR but Not T876AAR Mutant Can Be Activated by Casodex

It is known that casodex is a pure antiandrogen in LNCaP cells, indicating that casodex does not activate T876AAR *(19)*. Consistent with the previous report *(19)*, our data showed the transcriptional activity of T876AAR in response to casodex was similar to that of the wtAR (Fig. 3; lanes 8 to 9 with 2 to 3). By contrast, under the same concentration of casodex treatment, the C685YAR mutant showed a 2- to 4-fold higher transcriptional activity than that of wtAR or T876AAR (Fig. 3, lanes 5 to 6 vs 2 to 3 or 8 to 9). This result indicates that C685YAR is superior to T876AAR for detection of agonist activity from casodex.

C685YAR Showed Biphasic Response to Casodex but Not WtAR or T876AAR

Because of the antagonist activity of casodex to wtAR, casodex would compete with DHT and repress the DHT-induced transcriptional activity of AR. However, if significant agonist activity of casodex were to occur, we would expect to see a biphasic dose-responsive curve. As shown in Fig. 4A, casodex at 1–10 μ*M* repressed DHT-induced transcriptional activity of wtAR and of T876AAR in a dose-dependent manner (lanes 1–3 and 7–9). By contrast, a biphasic curve was observed in C685YAR with a combinational treatment with DHT and 1–10 μ*M* casodex (Fig. 4A, lanes 5 and 6), indicating that C685YAR is indeed more sensitive to the agonist activity from casodex than T876AAR even when DHT is present.

Suppression of DHT-Induced Transcriptional Activity of E708KAR Mutant by HF, E_2, and P

As our results show, the transactivation of E708KAR in response to DHT was similar to that of wtAR, but its transactivation by HF, E_2, and P was totally lost. We were interested to know whether these ligands could compete with DHT for transcriptional activation of E708KAR. As shown in Fig. 4B, HF at 0.1–1 μ*M* repressed DHT-induced transactivation in both wtAR and the E708KAR mutant (lanes 2 to 3 and 12 to 13). At a higher HF concentration (>10 μ*M*), HF activated, instead of repressed, transactivation in wtAR but not in the E708KAR mutant (lane 4 vs 14). Both E_2 and P added a small induction to the DHT-induced transactivation of wtAR (lanes 5–10). Under the same treatments, however, E_2 or P repressed DHT-induced transactivation of the E708KAR mutant in a dose-dependent manner (lanes 15–20). These data suggest that the E708KAR mutant is more sensitive to the antagonist activity of HF, E_2, and P.

Furthermore, Western blot analysis was performed to confirm that a similar expression level of AR protein was

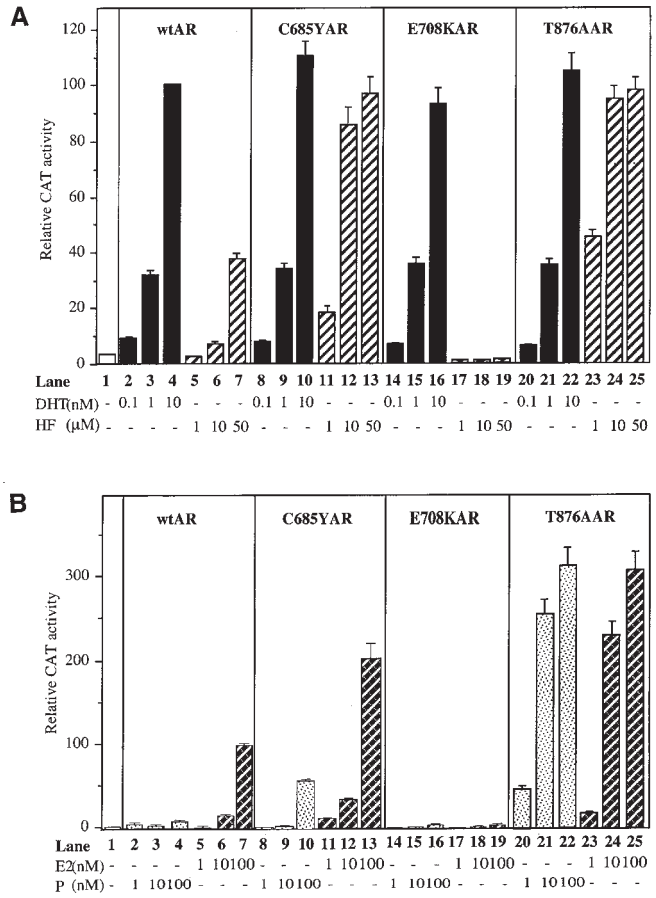

Fig. 2. The transcriptional activity of wtAR and the C685YAR, E708KAR, and T876AAR mutants, in response to increasing doses of various ligands. H1299 cells were cotransfected with AR expression vectors, the MMTV-CAT reporter, and β-galactosidase plasmids. The cells were then treated with **(A)** 0.1–10 n*M* DHT or 1–50 μ*M* HF, or **(B)** 1–100 n*M* E_2 or 1–100 n*M* P. The CAT activity was normalized to the β-galactosidase activity and expressed relative to that of wtAR either treated with 10 n*M* DHT in (A) or with 100 n*M* E_2 in (B).

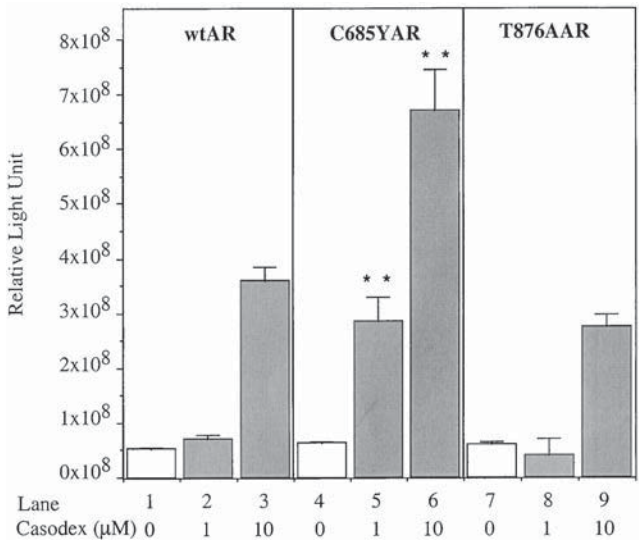

Fig. 3. The transcriptional activity of wtAR and the C685YAR and T876AAR mutants in response to increasing doses of casodex. PC-3 cells were transfected with AR expression vectors, the MMTV-AP reporter, and β-galactosidase plasmids. The cells were either not treated or treated with 1–10 μ*M* casodex. The alkaline phosphatase (AP) activity was normalized to the β-galactosidase activity and expressed as relative light units. Each bar represents the mean ± SD of three determinations. Student's *t*-test was used for statistical analysis compared with the same concentration of casodex treatment in wtAR. **, $p < 0.01$.

produced among different treatments. The mutations at C685Y, E708K, and T876A of AR did not influence protein stability. Although DHT could stabilize these AR proteins, the differences in expression level between different ligand treatments were less than 2-fold. Thus, the differences in transactivation observed were not owing to relative expression of AR (data not shown).

Inhibition of DNA-Binding Ability of E708KAR by E_2 and P

As shown in Fig. 5B, the E708KAR mutant bound to ARE with the same efficiency as the wtAR in the presence of DHT (lanes 1 and 5). This AR-ARE binding (solid arrows) was significantly inhibited by HF alone (Fig. 5A, lane 2). The addition of AR-specific antibody caused a supershift (open arrow) of the protein-DNA complex, indicating this was indeed a specific AR-ARE complex (Fig. 5A, lanes 3 and 6). More important, both E_2 and P inhibited E708KAR mutant binding to ARE but did not have a significant effect on wtAR-ARE binding (Fig. 5B, lanes 6 to 7 vs 2 to 3). These results demonstrated a strong correlation between ligand-regulated DNA-binding ability and the transcriptional activity of AR. Thus, the lack of transcriptional activity of the E708KAR mutant in response to E_2 and P could be owing to a loss of DNA-binding ability once the receptor binds the ligands.

Discussion

A method using chemical mutagenesis followed by yeast screening was employed to isolate AR mutants with divergent transcriptional activity in response to HF. The results from the yeast growth assay of transcriptonal activity of AR match well with those obtained from the reporter gene assay in mammalian cells, suggesting our assay based on the growth phenotype of yeast is good for isolating AR mutants with altered transcriptional activity in response to different treatments. Using this assay, two mutants, C685YAR and E708KAR, were isolated for further characterization at the levels of ligand binding, DNA binding, and transcriptional activation.

Because the crystallographic structure of progesterone receptor (PR) has been revealed and shown to have very high homology to AR, especially in the LBD, we would like to use the PR as a model to explain the potential effect of AR mutations on ligand function *(20,21)*. The E708KAR mutant, isolated four times in our screening, is of particular interest because the mutated site is located in helix 3, which

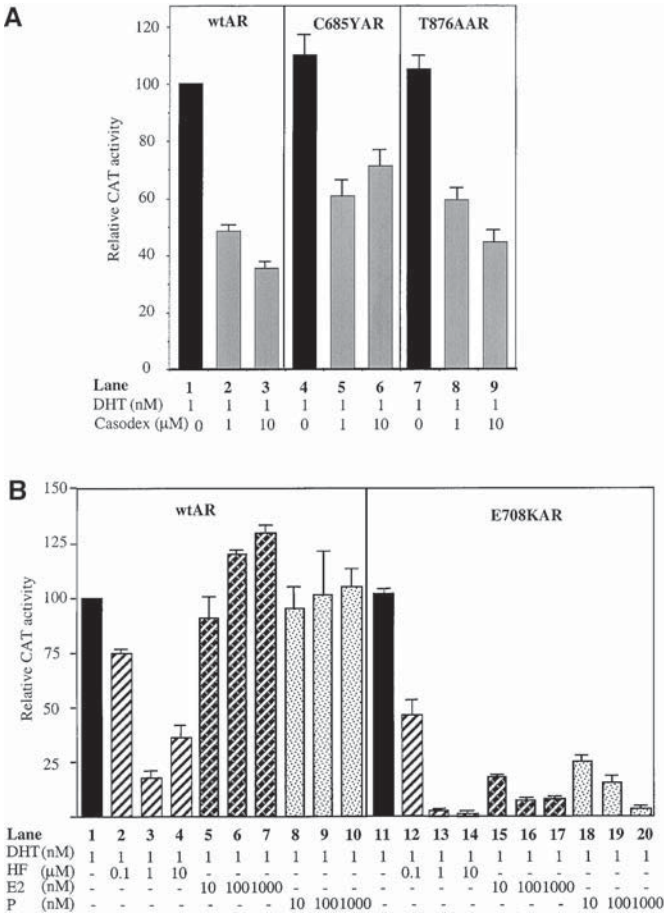

Fig. 4. Effects of various ligands on the DHT-mediated transcriptional activation of wtAR and the AR mutants. H1299 cells were cotransfected with expression vectors, the MMTV-CAT reporter, and β-galactosidase plasmids. The cells were then treated with **(A)** 1 n*M* DHT together with various doses of casodex, or **(B)** HF, E_2, and P. Lysate amounts used for CAT assays were normalized to the β-galactosidase activity of each sample. The CAT activity of wtAR treated with 1 n*M* DHT was expressed as 100%. Each bar represents the mean ± SD of three determinations.

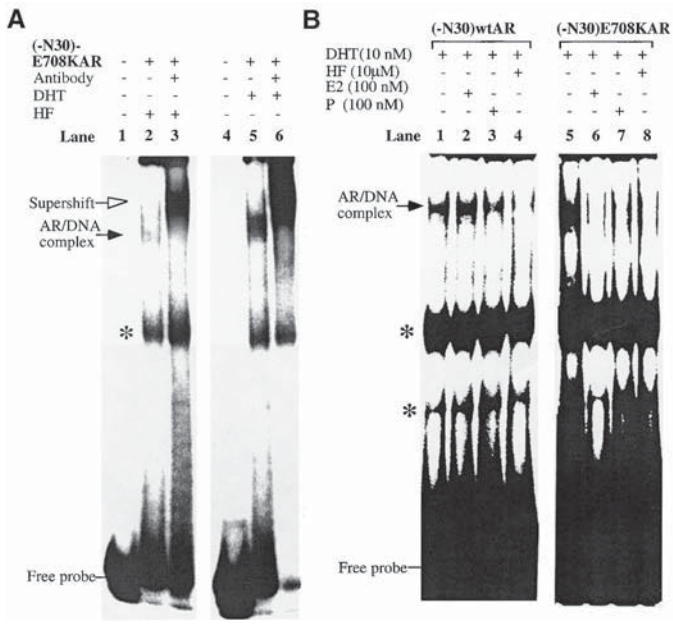

Fig. 5. Characterization of the in vitro DNA-binding properties of wtAR and the E708KAR mutant using electrophoretic mobility shift assay (EMSA). Recombinant wtAR and E708KAR mutant protein missing the first 30 amino acids of the N-termini were produced by baculovirus and then partially purified. The oligonucleotide of C3-ARE was used as a probe. The DNA-binding reaction of E708KAR mutant was carried out in the presence of **(A)** DHT or HF alone, or **(B)** in the presence of HF, E_2, or P to compete with DHT for AR binding. The position of the AR/DNA complex is indicated by the solid arrows and this AR/DNA complex was further supershifted by the AR-specific antibody CW2 as indicated by the open arrow. *, The positions of nonspecific binding.

is known to be involved in ligand-binding specificity (Fig. 1). For example, the residue 725 of PR, which is one of the three major residues that contact ligands through hydrogen bonding, corresponds to the residue 710 of AR (only two amino acids from 708). Interestingly, the C685Y mutation could also affect the AR residue 710. Alignment analysis indicated that residue 685 of AR corresponded to residue 700 of PR, which lies in helix 2 and is located directly behind residue 725 in the three-dimensional structure. Thus, mutations at residue 685 or 708 could play major roles in conformational change when AR is bound to individual ligands.

The transcriptional activity of the E708KAR mutant in response to DHT is very similar to that of the wtAR. The only difference between the E708KAR mutant and wtAR is the lack of E_2-, P-, or HF-induced E708KAR mutant transcriptional activity. Therefore, we were surprised to find that the E708KAR mutation could contribute to the development of the partial androgen-insensitive syndrome (PAIS) *(14)*. A possible explanation for the cause of this PAIS is in the interruption of the essential roles of the E_2- or P-mediated AR transactivation in the development of the normal male reproductive system. As our previous report suggested, ARA70, an AR coactivator, could enhance E_2-mediated AR transcriptional activity only in the wtAR, but not in the E708KAR mutant *(14)*. The failure of this E_2-AR-ARA70 pathway may account for the development of PAIS.

Another explanation is that E708KAR, once it binds to E_2 or P, could lose its DNA-binding ability, whereas this does not happen with wtAR. In our gel-shift experiments, higher than the required concentration of E_2 and P were used. The purpose was to shorten the incubation time for the ligands to replace DHT and to prevent proteins from degradation in DNA-binding reaction. Although the concentration of E_2 or P used did not mimic a physiological condition, there are clear differences in the DNA-binding capacity between wtAR and E708KAR under the same E_2 or P treatments. These differences could be a possible molecular mechanism caused by the phenotype of E708KAR mutant in patients with PAIS.

Although many mutant ARs have been identified, the isolation of C685YAR has not been reported previously. Introduction of the C685Y mutation into AR rendered the AR supersensitive to the agonist activity of antiandrogens and other related steroids. In the presence of DHT alone, C685YAR behaved just like wtAR. When DHT was combined with other ligands, only additional but not synergistic effects between ligands were observed in the C685YAR mutant (data not shown). This is probably the reason that the C685YAR mutation has never been discovered in clinical cases. Interestingly, changing amino acid 685 from cysteine to arginine, rather than tyrosine, reduced AR activity and caused PAIS in men *(22)*. These results suggest that residue 685 plays determining roles in the regulation of the transcriptional activity of AR. Different amino acid mutations at the same position may result in different phenotypes.

Comparing the responses to antiandrogens between C685YAR and T876AAR we observed several differences. First, even though both the C685YAR and T876AAR mutants could be transcriptionally activated by HF, E_2, and P, only T876AAR had a higher ligand-binding affinity to these ligands. This was surprising to us because sequence alignment indicated that AR has a cysteine at position 685, whereas all other classic steroid receptors have tyrosine at the corresponding position (Fig. 1). Changing cysteine to tyrosine at position 685 was expected to alter the ligand-binding characteristics of AR, causing it to become more ER- or PR-like, but our data did not support the assumption. Second, both C685YAR and T876AAR had a similar binding affinity to casodex but only C685YAR could be transcriptionally activated by casodex. These differences suggest that the transcriptional activation of C685YAR and T876AAR by various ligands could go through different mechanisms. Activation of T876AAR by HF, E_2, and P could be owing to an increase in ligand-binding affinity, whereas in the case of C685YAR, it could be owing to a productive conformational change, which may allow a induction of the activation domain of AR, creation of a new phosphorylation site, or enhancment of the ability to recruit different coactivators.

Although C685YAR has not been identified in prostate cancer tumors, it provides a powerful tool for analyzing agonist activity of antiandrogens. Clinically, prostate cancer patients with long-term casodex treatment could also develop antiandrogen withdrawal syndrome, indicating that casodex is not a pure antiandrogen. This result, together with our data showing that only C685YAR, not T876AAR, could be activated by casodex, provides evidence that C685YAR could be a better tool than T876AAR in determining agonist activity for the screening of pure antiandrogens.

Several other AR point mutations have also been isolated showing diverging transcriptional activity of AR in response to different ligands. For example, the T876AAR mutation is known to increase the ligand-binding affinity toward HF, E_2, and P *(16,18)*. The V715M mutation results in a higher sensitivity of AR to P, dihydroepiandrosterone, and androstenedione *(23)*. Another mutation, A725L, isolated from advanced prostate cancer, was found to show transcriptional activation that is inducible by E_2 *(24)*. The mechanisms behind the function of these mutations remain largely unknown.

In conclusion, we have isolated AR mutants with divergent transcriptional activity to HF. The C685YAR mutant renders AR supersensitive to the agonist activity of various ligands without changing the ligand-binding affinity. These characteristics make it a unique tool for screening pure antiandrogens that are greatly needed in the treatment of prostate cancer. The E708KAR mutant is the first mutant isolated to demonstrate a normal response to DHT in ligand-binding affinity and transcriptional activation, whereas its DNA-binding ability can be abolished in the presence of other ligands, a mechanism that could help explain the phenotype observed in PAIS patients with a natural E708KAR mutation.

Materials and Methods

Plasmid Construction

The reporter plasmid pARE-HIS3, in the backbone of pGAD 424 (Clontech), was constructed for AR mutant screening. It contains two copies of ARE oligonucleotides (5'-ACATAGTACGTGATGTTCTCAAGA-3') from rat prostate *C3* gene *(25)* upstream of the Gal 1 minimal promoter and a histidine gene as well as a leucine gene, for nutrient selection in yeast. The Gal 1 promoter and histidine gene was the *Bam*HI and *Sal*I fragment cut from the plasmid pRS315 (a gift from Dr. Randall R. Reed). For mammalian expression, AR cDNA fragments were inserted into pCMV vector, which contains the cytomegalovirus immediate early gene promoter in front of cloning sites. The plasmid pCMV-AR1, containing the full-length wtAR, was constructed by cloning the *Not*I fragment of pVL1393-AR into pCMV vector *(26)*. The plasmids pCMV-E708KAR and pCMV-C685YAR were constructed by replacing the 1-kb *Sal*I/*Kpn*I fragment of pCMV-AR1 with the 1-kb *Sal*I/*Kpn*I fragment of pG1-E708KAR and pG1-C685YAR, respectively. To construct the plasmid pCMV-876AR, the pCR2-876AR was made first by inserting the *Not*I/*Hinc*II fragment of pVL1393-AR into the plasmid pCR2-AR LBD (a gift from Dr. C. Kao). The plasmid pCR2-876AR was cut with *Bam*HI, which flanked the insert, blunt-ended, and then was cut with *Not*I to release the whole insert for ligation with the pCMV vector.

Chemical Random Mutagenesis

Hydroxylamine-based mutagenesis was performed as previously described *(27)*. Briefly, 100 μg of plasmid pG1-hAR was mixed with 5 mL of 1*M* hydroxylamine, incubated at 75°C for 75 min, and then purified by ethanol precipitation following phenol-chloroform extraction.

Yeast Strain

Two yeast strains, W303lb and ACY40, were gifts from Dr. Caplan *(28)*. The ACY40 strain is a W303lb with the integration of the reporter plasmid pPGKareLacZI at the *URA 3* locus.

Screening of AR Mutants

The lithium acetate transformation method was used to transform W303lb yeast cells with the reporter plasmid pARE-HIS3, together with the hydroxylamine-treated pG1-hAR (20 μg), which contains tryptophan nutrient selection marker *(29)*. Briefly, the cells were plated onto –2 SD (with amino acid mixtures lacking leucine and tryptophan) plates containing 2% dextrose and 0.67% yeast nitrogen base, and were grown at 30°C for 2 d. The colonies were then replica plated on –3 SD plates (with amino acid mixtures lacking leucine, tryptophan, and histidine), containing 2% dextrose and 0.67% yeast nitrogen base, with the addition of 20 m*M* 3-aminotriazole (Sigma) and various concentrations of ligands. The plates were incubated at 30°C for 2 d. The colonies that showed growing phenotypes different from wtAR were isolated.

To confirm that the phenotypes of yeast clones were caused by mutated ARs, a second plate growth assay was performed. AR plasmids were recovered from the colonies and retransformed into W303lb containing the reporter plasmid pARE-HIS3. In addition, DNA sequencing throughout the entire AR coding region was performed to identify point mutations.

Ligand-Binding Assays

Whole-cell-ligand binding assays were performed as previously described with some modification *(26)*. Briefly, H1299 cells on 60-mm dishes were transfected with the AR expression vectors pCMV-wtAR, pCMV-E708KAR, pCMV-C685YAR, and pCMV-T876AAR using the calcium phosphate method as described. After 36 h of transfection, the cells were harvested, washed, and then incubated with 200 μL of 2.5 n*M* [^{3}H]-R1881 (NEN) in the absence or presence of unlabeled ligands. After 1 h of incubation, the cells were washed with phosphate-buffered saline and then resuspended in 200 μL of ligand-binding buffer (20m *M* HEPES, pH 7.5; 1 m*M* EDTA; 0.4 *M* KCl; 1m*M* dithiothreitol [DTT], 10% glycerol; 0.1 m*M* phenylmethylsulfonyl fluoride [PMSF]; 10 m*M* sodium molybdate). The cells were lysed through three freeze-thaw cycles. Unbound R1881 was removed by incubation with 150 μL of hydroxyapatite (60% [v/v] in ligand-binding buffer; Bio-Rad) and then centrifuged. Radioactive R1881 retained in hydroxyapatite was determined by liquid scintillation counting.

Transfection and Reporter Gene Assay

H1299 cells were cotransfected with calcium phosphate reagents with 3 μg of the AR expression vectors (pCMV-wtAR, pCMV-E708KAR, pCMV-C685YAR, and pCMV-T876AAR), 4 μg of the reporter plasmid pMMTV-CAT (Pharmacia), and 1 μg of the internal control lacZ expression vector pCMVβ (Clontech). Extracts were prepared from transfected cells and assayed for CAT and β-galactosidase activities as described elsewhere *(30)*.

Transfection and Phosphatase Assay

To use AP as a reporter gene, we constructed the pMMTV-AP reporter. The DNA fragment containing MMTV LTR was released by digestion of the pMSG plasmid (Pharmacia) with *Hind*III and *Sma*I, and cloned into the pSEAP2 vector (Clontech) between the *Hind*III and *Nru*I sites.

PC-3 cells were transfected with 2 μg of AR expression vectors, 2 μg of pCMVβ, and 6 μg of the reporter plasmid pMMTV-AP by electroporation. After 16 h, the medium was refreshed and the cells were treated with ligands for 16 h. Subsequently, 50 μL of medium was taken for AP-light chemiluminescent assay (Tropix) by top counter (Packard). Cells were harvested and extracts were assayed for β-galactosidase activity.

Western Blot Analysis

H1299 cells were cotransfected with AR expression vectors and pCMV-β. The amounts of lysate used for Western blotting were normalized to the β-galactosidase activity in each sample. Normalized lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to Immobilon membrane (Millipore). The AR bands were detected by anti-AR polyclonal antibody NH27, followed by incubation with the secondary antibody AP-conjugated goat antirabbit immunoglobulin (Pharmingen), and developed using a color substrate kit for AP (Bio-Rad). The AR expression levels were quantified by using a digital camera (Fotodyne) with Collage image analysis software (Fotodyne).

Expression of AR Protein

AR proteins were overexpressed using a Baculovirus vector system (PharMingen) *(26)*. Two plasmids, both missing the first 30 amino acids on the N-terminal end of AR, pVL(-N30)-wtAR, and pVL(-N30)-E708KAR, were constructed in the vector pVL1393 (PharMingen) for baculovirus expression. The recombinant baculovirus producing the wtAR or E708KAR was generated by homologous recombination after cotransfection of *Spodoptera frugiperda* 9 (Sf9) insect cells with the BaculoGold linearized baculovirus DNA (PharMingen) and the baculovirus expression plasmids using the BaculoGold transfection kit (PharMingen). To produce the AR proteins, Sf9 cells infected with the recombinant baculovirus were harvested, resuspended in His-binding buffer (20 m*M* HEPES, pH 7.5; 0.5 *M* NaCl; and 0.1 m*M* PMSF), lysed through five freeze/thaw cycles, and centrifuged. The supernatant was further purified by histidine column chromatography following the manufacturer's protocol

(Pharmacia). The AR proteins were eluted from the histidine column using 0.2 *M* EDTA and then dialyzed against EMSA binding buffer (20 m*M* HEPES, pH 7.9; 60 m*M* KCl; 1 m*M* EDTA, 1 mM DTT) with 10% glycerol. Protein concentration was quantified by the Bradford method using a Protein Assay kit (Bio-Rad).

Electrophoretic Mobility Shift Assay

EMSA was performed using purified AR protein and using γ-[^{32}P]-end-labeled C3-ARE as the probe. Baculovirus-expressed wtAR or mutant AR protein (30 μg) was incubated with C3-ARE probe (1×10^5 cpm) in the presence of various ligands in 20 μL of EMSA binding buffer at room temperature for 15 min. For the antibody supershift assay, 2 μL of the anti-AR polyclonal antibody, CW2, was added to the reaction at 25°C for 15 min, before loading onto a 4% native polyacrylamide gel. After electrophoresis, the gel was dried and exposed to Kodak X-AR film.

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