

The Effect of Androgens and 17 β -estradiol on the Androgen Receptor Transcriptional Activity in the Presence of the Androgen Receptor co-activator ARA₇₀ in Human Prostate DU145 Cells

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Summary

The androgen receptor (AR) is a member of the steroid receptor superfamily that plays an important role in male sexual differentiation and prostate cell proliferation [1, 2]. Mutations or abnormal expression of AR in prostate cancer can play a key role in the process that changes prostate cancer from an androgen-dependent to an androgen-independent stage [3, 4]. Using a yeast two-hybrid system, we were able to isolate a ligand-dependent AR-associated protein (ARA₇₀), which functions as a coactivator to enhance an additional 10-fold AR transcriptional activity in the presence of 10⁻¹⁰ M dihydrotestosterone (DHT). Furthermore, the transcriptional activity of AR can also be induced by 10⁻⁸ M 17 α -estradiol (E2), but not 10⁻⁶ M diethylstilbestrol (DES), a more potent synthetic estrogen, in the presence of ARA₇₀ in human prostate DU145 cells. These data suggest that AR may need coactivator(s) such as ARA₇₀ for optimal androgen activity, and the ARA₇₀ can also function as an enhancer to increase androgenic activity on 17 β -estradiol.

Results and Discussion

To further understand the mechanism of androgen-AR action, we have applied a yeast two-hybrid system using the GAL4 DNA binding domain

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(GAL4DBD) fused with the human AR peptide (amino acids 595 to 918) as bait (GAL4AR) to isolate a cDNA encoding ARA₇₀ which interacts specifically with AR. In this system, yeast will survive when GAL4AR is co-expressed with ARA₇₀ in the presence of DHT. Neither GAL4AR nor ARA₇₀ was active when ARA₇₀ was expressed alone or when ARA₇₀ was co-expressed with GAL4RXR or GAL4TR4 (GAL4 fusion proteins with two other members of the steroid receptor superfamily). These data [5], therefore, clearly suggested that ARA₇₀ can interact specifically with AR in the yeast cells.

We then tested whether the interaction of ARA₇₀ with AR in yeast was ligand-dependent. DHT (5×10^{-10} M) can promote the interaction between ARA₇₀ and GAL4AR. Testosterone (T), a less potent androgen in the prostate, can also promote this interaction at higher concentrations (greater than 5×10^{-9} M); glucocorticoid (dexamethasone) had no activity even at a very high concentration (10^{-5} M). Surprisingly, we found that 17β -estradiol (5×10^{-8} M), a natural estrogen, can also induce the interaction between ARA₇₀ and GAL4AR, but DES, a nonsteroidal but more potent estrogen was unable to promote this interaction even at pharmacological concentrations (10^{-6} to 10^{-5} M) (Yeh and Chang, manuscript submitted).

To further confirm that the interaction that occurred in yeast cells is due to a direct interaction between AR and ARA₇₀, we applied an *in vitro* immunoprecipitation assay with an anti-AR antibody (CW2). We demonstrated that CW2 can co-precipitate AR and ARA₇₀ when *in vitro* transcribed/translated full-length human AR and ARA₇₀ were incubated with it in the lysate mixture. This precipitation is specific, as CW2 did not precipitate the ARA₇₀ in the absence of AR and CW2 did not precipitate two other proteins (RXR and TR4 orphan receptors) incubated with AR. Far-Western assay also demonstrated that ARA₇₀ can bind to immobilized AR but not other control proteins. Together, these data indicate that the association is due to a direct interaction between AR and ARA₇₀[5].

To test whether ARA₇₀ will affect the AR transcriptional activity, DU145 cells (with passage number above 200) were co-transfected with ARA₇₀ and AR under eukaryotic promoter control. Ligand-free AR has a minimal MMTV-ARE CAT reporter activity with or without the presence of ARA₇₀. Addition of DHT results in a 6-fold increase of AR activity. Furthermore, this transcriptional activity can be increased to 58 (± 3.2)-fold (mean \pm SEM; $n = 4$) by the co-transfection of ARA₇₀ cDNAs in a dose-dependent manner. The induced activity reached a plateau when 4.5 μ g of co-transfected ARA₇₀ cDNA. Additional ARA₇₀ beyond 4.5 μ g (up to 6 μ g) did not affect the induced activity of AR in DU145 cells. To rule out any indirect effects on the basal activity of the MMTV-ARE CAT reporter, we removed the ARE DNA fragment from our reporter (MMTV- Δ ARE-CAT). The results showed that ARA₇₀ induced no activity on this reporter in the presence or in the absence of DHT⁵. Together, these data strongly suggest that stimulation of AR transcriptional activity by ARA₇₀ may occur through a ligand-bound AR.

We also replaced MMTV-ARE CAT with PSA-ARE CAT. This PSA-ARE CAT encompasses the promoter of PSA gene from -2.8 kb to the translational start site. Our data indicate ARA₇₀ also enhanced AR transcriptional activity on the PSA reporter gene (Fig. 1). Furthermore, when we replaced DU145 cells with CHO cells, which express a relative abundance of ARA₇₀, we found that

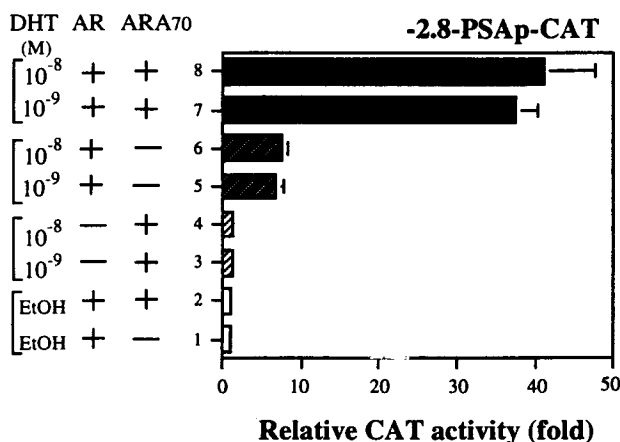


Figure 1. DHT can further enhance the transcriptional activity of AR in the presence of ARA_{70} on the PSA promoter. The fixed amounts of AR (1.5 μ g) and ARA_{70} (4.5 μ g) were transfected into DU145 cells in the absence or presence of 10^{-9} M and 10^{-8} M DHT for CAT assay. The DU145 cells were plated in 60-mm diameter petri dish at a density of 3.5×10^5 cells per dish. The expression plasmids were transfected into cells by the calcium phosphate method [11]. In each transfection, 3.5 μ g reporter (PSA-ARE CAT) was co-transfected into the DU145 cells. All data were the average from results of three independent experiments.

although the exogenously transfected ARA_{70} did not show a dramatic effect on induction of AR transcriptional activity, the transfection of antisense ARA_{70} did show the ability to partially block the AR transcriptional activity (Yeh and Chang, manuscript in preparation).

The effect of ARA_{70} on transactivation of AR bound to different concentrations of steroids in DU145 cells was also tested. Whereas 10^{-10} M DHT maximized the induced transcriptional activity of AR, T needed a 10-fold higher concentration (10^{-9} M) for maximum activity. These results are consistent with the data generated from yeast cells⁵ and previous reports [6, 7], which indicated DHT is a more potent androgen in the prostate. In fact, the greater potency of DHT to modulate the interaction between AR and ARA_{70} may actually provide another reason why DHT is a more potent androgen in prostate. Recently, our data in DU145 cells also indicate that 17β -estradiol, at a concentration of 10^{-8} M and higher, can clearly induce the transcriptional activity of AR only in the presence of ARA_{70} (Fig. 2, lane 12), but not in the absence of ARA_{70} (data not shown). Again, DES (a more potent synthetic estrogen) shows no such induced activity, even at a pharmacological concentration (10^{-6} M) (Fig. 2, lane 23).

To rule out the possibility that the induced CAT activity of the MMTV-ARE CAT construct may be mediated from endogenous ER instead of transfected AR in the presence of 17β -estradiol and ARA_{70} , we replaced AR with ER in our MMTV-ARE CAT assay. Our data clearly indicate that only AR, but not ER, can significantly induce the CAT activity in the presence of 17β -estradiol and ARA_{70} (Yeh and Chang, manuscript submitted).

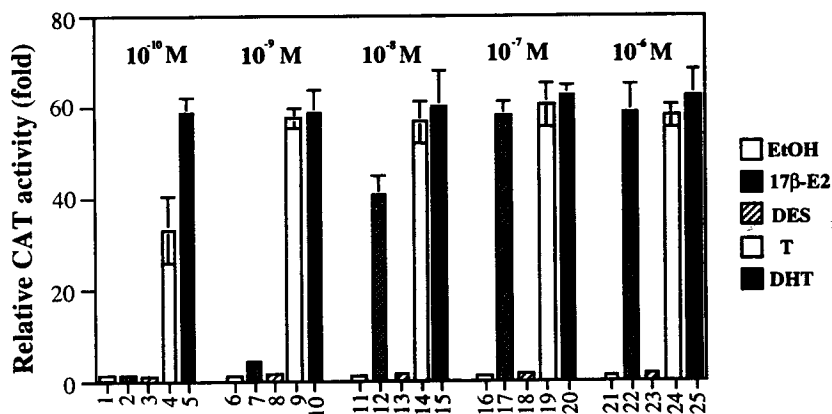


Figure 2. Effects of DHT, T, DES, and 17 β -E2 on the AR transcriptional activity in the presence of ARA₇₀ in DU145 cells. The amount of hAR (1.5 μ g) and ARA₇₀ (4.5 μ g) that reached the maximal AR induction level in DU145 cells were fixed in the assay in the presence of a serial concentrations of DES, 17 β -E2, T, and DHT. DU145 cells were plated in 60-mm diameter petri dish at a density of 3.5×10^5 cells per dish and the MMTV-LTR CAT was used as a reporter for the assay of transcriptional activity. The expression plasmids were transfected into cells by the modified calcium phosphate method [11].

Our data demonstrate that a protein such as ARA₇₀ can function as an enhancer to increase the androgenic activity on 17 β -estradiol. These findings may allow us to reconsider the definition of so-called “androgenic” or “estrogenic” compounds. Although we still cannot explain why ARA₇₀ can only enhance androgenic activity on 17 β -estradiol but not DES, our findings may provide one explanation about why DES, but not 17 β -estradiol, has fewer side effects when used by clinicians to treat prostate cancer patients [8, 9].

Furthermore, using the same DHT-AR bait, we were able to isolate and characterize another potential AR-associated protein, ARA₆₂. Unlike ARA₇₀, ARA₆₂ did not associate with E2-AR in a yeast two-hybrid interaction system (Fig. 3A). This result revealed that the conformation of E2-AR, which can not be recognized by ARA₆₂, may be different from that of DHT-AR. Partial protease digestion further supports that DHT-AR and E2-AR may have different conformations (Fig. 3B). Further crystallography may allow us to detect the conformational differences between DHT- and E2-AR/ARA₇₀.

Together, our finding that E₂ can activate androgen target genes in the presence of both AR and ARA₇₀, but not in the presence AR only, indicates that: (1) the receptor specificity and biological diversity of the steroid receptor family can be modulated at the level of co-factors; (2) the conformation of E2-AR may be different from that of DHT-AR.

The enhancement of AR transcriptional activity from 6-fold to 58-fold by ARA₇₀ may expand androgen activity in the prostate that androgen-AR alone cannot reach. Since we detected ARA₇₀ in AR-positive LNCaP prostate cancer cells, but not in AR-negative DU145 cells, it will be important to determine if the expression of ARA₇₀ and its ability to interact properly with androgen-AR

change during the progression of prostate cancer from an androgen-dependent to an androgen-independent state.

Several co-factors have been demonstrated to interact with other steroid receptors in a ligand-dependent or ligand-independent manner [10]. To date, none of these proteins have been reported to specifically enhance AR-mediated transcriptional activity; therefore, it is likely that ARA₇₀ has a different mechanism for interacting with AR.

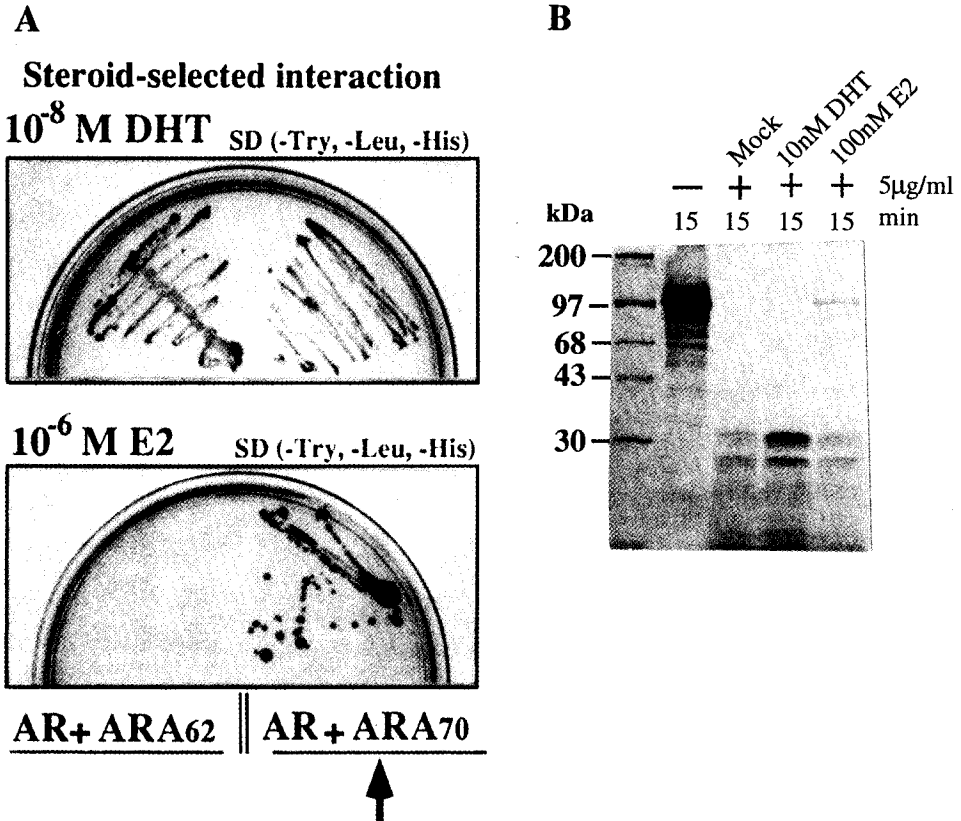


Figure 3. The DHT-AR may have different conformation from that of E2-AR (A) Comparing the effects of DHT and 17 β -estradiol (E2) on the interaction of ARA₇₀ or ARA₆₂ with GAL4AR by plate nutritional selection in the yeast Y190⁵. The AR bait and ARA₇₀ (or ARA₆₂) co-transformed yeast cells were selected for growth on plates with 30 mM 3-aminotriazole and either DHT or E2 but without histidine, leucine, or tryptophan. Data were reproducible from two independent transformations. (B) Partial protease conformation assay. Five- μ l aliquots of *in vitro* translated [³⁵S]methionine AR were incubated with 10 nM DHT or 100 nM E2. 0.5 μ l of the Type III trypsin (50 μ g/ml, diluted in H₂O, Promega) was added on the wall of each microcentrifuge tube, short spin for 10 seconds, and the reactions were started simultaneously for 15 mins at 25 C. Before loading, the samples were heated at 95 C for 5 mins, and loaded onto a 10%-12.5% two-layer gradient SDS-polyacrylamide gel.

In summary, the demonstration that ARA₇₀ can function as a co-activator to enhance the AR transcriptional activity and that it might function as an enhancer to increase androgenic activity on 17 β -estradiol may help us to better understand the molecular mechanism of sex hormones and their receptors.

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