

Differential Induction of Androgen Receptor Transactivation by Different Androgen Receptor Coactivators in Human Prostate Cancer DU145 Cells

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Recently identified androgen receptor (AR) coactivators were used in this study to determine whether the specificity of sex hormones and antiandrogens could be modulated at the coactivator level. We found that ARA70 is the best coactivator to confer the androgenic activity on 17 β -estradiol. Only ARA70 and ARA55 could increase significantly the androgenic activity of hydroxyflutamide, a widely used antiandrogen for the treatment of prostate cancer. None of the AR coactivators we tested could significantly confer androgenic activity on progesterone and glucocorticoid at their physiological concentrations (1–10 nM). We also found that ARA70, ARA55, and ARA54, but not steroid receptor coactivator-1 (SRC-1) and Rb, could significantly enhance the Δ^5 -androstenediol-mediated AR transactivation. Furthermore, in comparing the relative specificity of these coactivators to AR in DU145 cells, our results suggested that ARA70 has a relatively higher specificity and that SRC-1 can enhance almost equally well many other steroid receptors. Finally, our data demonstrated that AR itself and some select AR coactivators such as ARA70 or ARA54 could, respectively, interact with CBP and p300/CBP-associated factors that have histone acetyltransferase activity for assisting chromatin remodeling. Together, our data suggest that the specificity of sex hormones and antiandrogens can be modulated by some selective AR coactivators. These findings may not only help us to better understand the specificity of the sex hormones and antiandrogens, but also facilitate the development of better antiandrogens to fight the androgen-related diseases, such as prostate cancer.

Key Words: Androgen receptor; coactivator; steroid specificity; 17 β -estradiol; antiandrogen; Δ^5 -androstenediol; p300/CBP-associated factor.

Introduction

The androgen receptor (AR) functions primarily as a ligand-activated transcription factor. On ligand binding, AR binds to androgen response elements and consequently activates its target genes (1,2). The functionally activated AR has a pivotal role in male sexual differentiation and prostate cell proliferation (3).

As a member of the nuclear receptor superfamily, AR shares a common structure organization with other nuclear receptors. In general, a nuclear receptor has an N-terminal transactivation domain, a DNA-binding domain (DBD), a hinge region, and a C-terminal ligand-binding domain (LBD). The crystal structure of the LBD of several nuclear receptors reveals that the ligand is almost entirely buried within the conserved core of helices 3, 7, and 10 (4,5). Currently, numerous nuclear receptor coactivators have been identified. Nuclear receptors may utilize these coactivators to enhance their transactivation, possibly involving allosteric alterations in helix 12 domain. These coactivators may also function as a bridge factor between the nuclear receptor and the basal transcription factor complex to promote changes in the chromatin structure.

Because of the high homology of the receptor interaction domain (RID) and other functional domains, some of the coactivators have been grouped as the SRC/p160 family, which includes steroid receptor coactivator-1 (SRC-1) (6), GRIP1/TIF2 (7,8), and RAC-3/ACTR/P/CIP (9–11). In addition, other receptor coactivators, such as TIF1, RIP140, TAFII30, PGC-1, a small nuclear ring-finger protein (SNURF), and a novel nuclear protein kinase (ANPK), have also been identified (12–17). Using the C-terminal domain of AR as bait, we have isolated the first AR coactivator, an AR-associated protein, ARA70, that can enhance AR transactivation an additional 3- to 10-fold in human pros-

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tate cancer cells (18). Recently, we were able to isolate and characterize several additional coactivators including ARA24, ARA55, ARA54, ARA160, and Rb (19–23). While most of these coactivators have been shown to bind multiple receptors, a few may possess some specificity. In addition, some of the coactivators have been identified as having intrinsic histone acetyltransferase (HAT) activity or could associate with proteins possessing HAT activity (24–27). However, the functional correlation of different coactivators with HAT activity and chromatin remodeling remains to be elucidated.

To date, prostate cancer has become the most frequently diagnosed neoplasm in the United States and the second leading cause of cancer-related death in American men (28). Androgens and AR play important roles in the progression of this malignancy (29). In prostate cancer patients undergoing androgen ablation therapy, the functions of testosterone and 5 α -dihydrotestosterone (DHT) are inhibited, but other compounds, such as antiandrogens, adrenal androgenic compounds, or other steroids, may be able to stimulate AR transactivation during therapy. Indeed, one of our previous reports indicated that the transfection of ARA70 could induce AR transactivation in the presence of 17 β -estradiol (E₂) (30). Mutagenesis studies further confirm that the amino acid 708 (Glu), located in helix 3 of AR, plays an essential role in mediating the E₂-AR-ARA70 pathway. Although the crystal structure of AR has not been elucidated, our data highlight the importance of helix 3 of AR in distinguishing the DHT- and E₂-mediated AR transactivation (30). Our recent reports also found that ARA70 could confer higher agonist activity on antiandrogens (31,32), or Δ^5 -Androstene-3 β ,17 β -diol (Adiol) in AR transactivation (33).

Three AR-LBD-associated coactivators identified in our laboratory are ARA70, ARA55, and ARA54. These ARAs all have different RIDs, transactivation domains, and signature functional domains that place these three AR coactivators outside of the common SRC/p160 family (7–12,19–21). Therefore, it will be of great interest to compare the functions of these different ARAs with the members of the SRC/p160 family. Here, we systematically compare the relative effects of various AR coactivators, ARA70, ARA55, ARA54, Rb, and SRC-1 on different hormone-mediated AR transactivations. Our results suggest that only select coactivators can modulate the androgenic activity of antiandrogen, E₂, and Adiol on AR. Furthermore, the specificity of these coactivators toward classic steroid hormone receptors was also investigated.

Results

DHT-Mediated AR Transactivation in the Presence of Different AR Coactivators

Although SRC-1 has been reported as an effective steroid receptor coactivator (6), results from DU145 cells indicate SRC-1 is a relatively weak AR coactivator. Previous reports

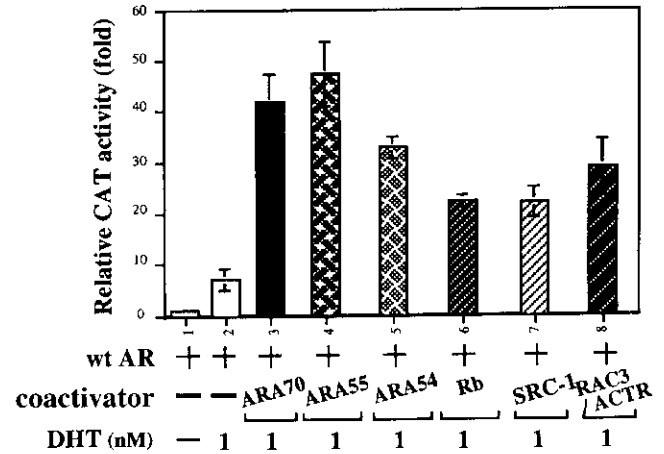


Fig. 1. Characterization of DHT effect on AR-mediated transactivation in the presence or absence of different ARAs in prostate cancer DU145 cells. One and a half micrograms of human AR alone or with 4.5 μ g of different AR coactivator were transfected into DU145 cells. Cells were treated with 1 nM DHT after 24 h of transfection. The transfection efficiency was normalized by the β -gal activity. Relative CAT activity was calculated by the quantitation of PhosphorImager (molecular dynamics). Data represent an average \pm SD of three independent experiments.

also indicated that SRC-1 had only a two-fold coactivator effect in CV-1 cells (9). Therefore, it will be very important to systematically compare the relative strengths of different coactivators under the same conditions. Among the cell lines we tested (CHO, DU145, LNCaP, PC-3, HeLa, and MCF7), the human prostate cancer cell line, DU145, was used because of the low activity of AR transactivation in the absence of exogenous AR coactivators.

To compare the relative enhancement of DHT-mediated AR transactivation with different AR coactivators, human AR and all available AR coactivators (ARA70, ARA55, ARA54, Rb, SRC-1, and RAC3) were inserted into the pSG5 expression vector to obtain the same transfection efficiency. As shown in Fig. 1, when human AR and/or individual AR coactivators were transiently expressed in DU145 cells without adding DHT, there was no AR transactivation. However, AR transactivation could be induced to five- to seven-fold when AR was expressed in the presence of 1 nM DHT (Fig. 1, lane 1 vs. 2). The addition of various AR coactivators, at a 1:3 AR:AR coactivator ratio, could further enhance the AR transactivation (5- to 7-fold without coactivator) to 22- to 45-fold in the following order: ARA55 (45-fold) > ARA70 (40-fold) > ARA54 (33-fold) > RAC3 (28-fold) > Rb (25-fold) > SRC-1 (23-fold). Together, these data suggest that ARA55 and ARA70 are the two most effective coactivators for DHT-mediated AR transactivation in DU145 cells.

Interactions Between AR and ARA70, ARA55, and ARA54 Are Androgen-Dependent

Our previous data showed that AR could interact with several AR coactivators in the presence of testosterone or

DHT in the yeast two-hybrid assay (18,20,21). To investigate whether the interaction that occurred in yeast cells was due to a direct interaction with AR, coimmunoprecipitation assays were performed using an *in vitro* transcription/translation system that expressed AR and His-Tag fusion with ARA55, ARA54, or ARA70. A polyclonal anti-His-tag antibody was employed for the coimmunoprecipitations, and the resulting immune complexes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 2A (lanes 1, 4, and 7), the lysate containing individual His-Tag fusion ARA was immunoprecipitated using the polyclonal anti-His-tag antibody. The AR protein could be clearly detected in association with individual ARAs in the presence of 10^{-8} M DHT (Fig. 2A; lanes 3, 6, and 9). Furthermore, in the absence of DHT, AR could not be precipitated by ARA70, ARA55, or ARA54 (Fig. 2A; lanes 2, 5, and 8). By contrast, our result showed AR could not pulldown the *in vitro* transcribed/translated SV40 large T-antigen (Tag) in the presence of DHT (data not shown), suggesting that the interactions between DHT-AR and ARAs are specific.

We then applied a mammalian two-hybrid assay to confirm this DHT-dependent interaction between AR and ARAs *in vivo*. DU145 cells were cotransfected with a plasmid encoding the LBD of wild-type AR (wtAR) fused to the GAL4 DBD (GALAR_{LBD}) and a plasmid encoding ARA55, ARA54, or ARA70 fused to the activation domain of VP16. Interaction was estimated by determining the level of luciferase activity from the reporter plasmid, and SV40 Tag was used as a negative control (Fig. 2B). In the absence of androgen, the combination of GALAR_{LBD} and individual VP16 fused-ARA showed a basal activity (Fig. 2B; lanes 1, 3, and 5). A significant level of luciferase activity was induced by the cotransfection of GALAR_{LBD} with VP16-ARA70 or VP16-ARA54, only in the presence of 1 nM DHT. The induction of VP16-ARA55 was not as high as ARA70 and ARA54, but still above three-fold. Together, results from the yeast two-hybrid assay, coimmunoprecipitation, and mammalian two-hybrid assay all indicate that the specific interaction between ARAs and AR is an androgen-dependent process.

E₂-Mediated AR Transactivation in Presence of Different AR Coactivators

Previous data using the mouse mammary tumor virus-androgen response element-chloramphenicol acetyltransferase (MMTV-ARE-CAT) reporter system have shown that 10 nM E_2 could further enhance AR transactivation in the presence of ARA70 (30). We were interested in determining whether this ARA70-mediated E_2 -AR new pathway could also occur with other AR coactivators, such as ARA55, ARA54, RB, SRC-1, and RAC3/ACTR. As shown in Fig. 3, we found that 10 nM E_2 could not significantly enhance the AR transactivation in the absence of AR coactivators (Fig. 3, lane 2 vs 1). Coexpression of ARA70

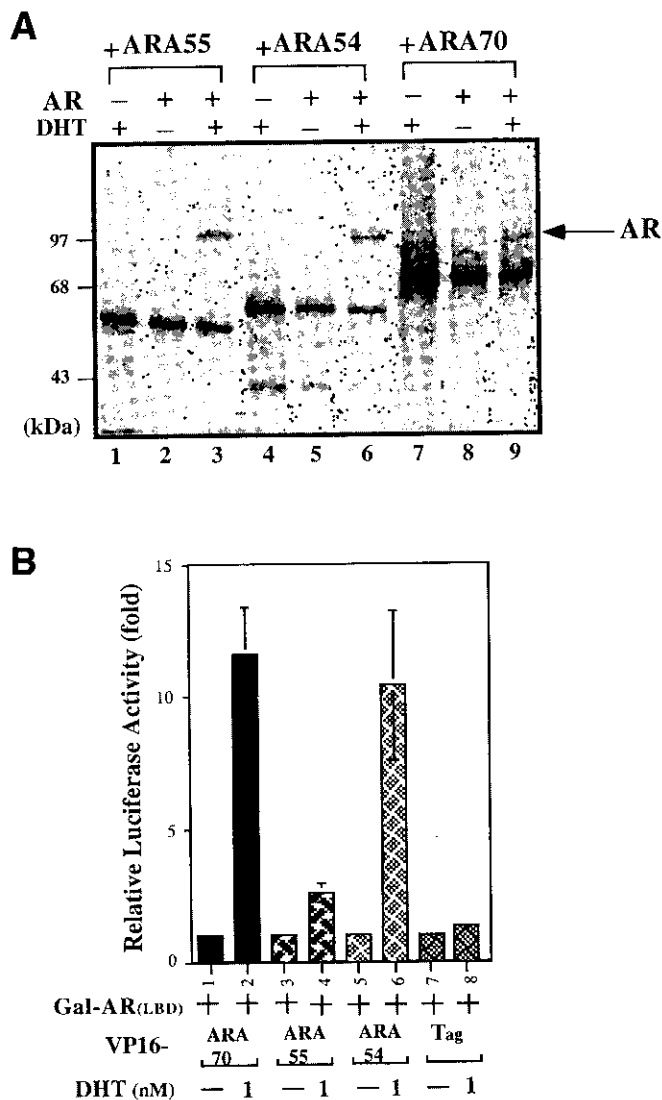


Fig. 2. (A) Coimmunoprecipitation of AR and AR coactivators. The *in vitro* translated pET-ARA70, ARA55, ARA54, and AR incubated in the presence or absence of 10^{-8} M DHT, are shown in lanes 1–9. The polyclonal anti-His-tag antibodies were used for coimmunoprecipitation and 10 μ L of protein A/G-Sepharose beads were applied to precipitate the protein-antibody complex. Molecular size markers are in kilodaltons. (B) Mammalian two-hybrid assay. DU145 cells were cotransfected with 3 μ g of GALAR_{LBD} encoding the LBD of wtAR fused to the GAL4LBD and 4.5 μ g of VP16-ARA70, ARA55, and ARA54 encoding the cDNA of individual coactivator fused to the activation domain of VP16. Interaction was estimated by determining the level of luciferase activity from 3 μ g of the reporter plasmid pGSLUC in the presence of 1 nM DHT.

could then enhance the AR transactivation 2– to 4-fold at 1 nM E_2 and above 20-fold at 10 nM E_2 . Among the other AR coactivators we tested, only SRC-1 was able to enhance E_2 -mediated AR transactivation near seven- to eight-fold at 10 nM E_2 (Fig. 3, lane 2 vs 16). Other coactivators only showed very marginal induction (two- to three-fold) in the presence of 10 nM E_2 . In addition, the AR target reporter can not be activated in the absence of AR (Fig. 3; lanes 5,

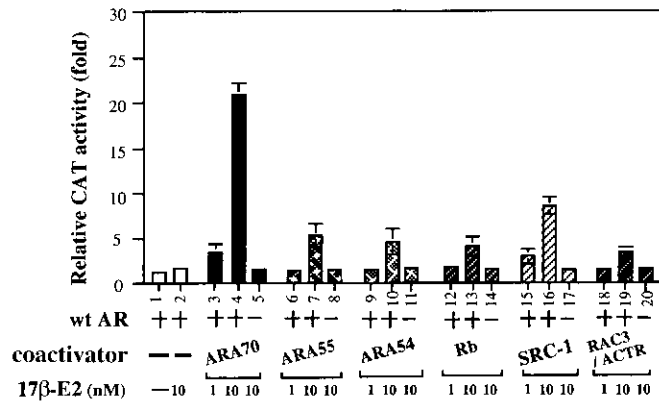


Fig. 3. Characterization of E_2 effect on AR-mediated transactivation in the presence or absence of different ARAs in prostate cancer DU145 cells. DU145 cells were cotransfected with 3.5 μ g of MMTV-CAT and 1.5 μ g of pSG5-AR in the presence or absence of 5 μ g AR coactivators with 1 or 10 nM E_2 treatment. The CMV- β -gal construct was used as an internal control, and the relative CAT activity was normalized by the β -gal activity. Data represent an average \pm SD of four independent experiments.

8, 11, 14, 17 and 20), suggesting that the presence of AR is essential for the E_2 -AR-ARAs-mediated transactivation.

Overall, our results indicated that there is a significant difference in the enhancement effect of DHT (Fig. 1) and E_2 -mediated (Fig. 3) AR transactivation by various coactivators. ARA70 and SRC-1 may represent significant coactivators that mediate the E_2 -AR pathway in DU145 cells.

Effects of Testosterone-, Progesterone-, or Dexamethasone-Mediated AR Transactivation in Presence of Different AR Coactivators

We then extended our study to other classic steroid hormones, such as testosterone, progesterone, and dexamethasone. As expected, testosterone and DHT had quite similar enhancement effects that could further induce AR transactivation in the presence of various AR coactivators with a similar order: ARA55 > ARA70 > ARA54 > Rb > SRC-1 (Fig. 4). However, there were only slight inductions of AR transactivation with various AR coactivators in the presence of 10 nM of progesterone or dexamethasone, although progesterone at a much higher concentration of 100–1000 nM, but not dexamethasone, could start to induce AR transactivation with ARA70 (data not shown). Our data, therefore, again show contrasting results among several well-known steroid hormones (DHT/T, E_2 , progesterone, and dexamethasone) with various AR coactivators.

Hydroxyflutamide-Mediated AR Transactivation in Presence of Different AR Coactivators

The partial agonist activity of antiandrogens, such as hydroxyflutamide (HF), during androgen ablation therapy of prostate cancer has been proposed as one of the possible reasons that most prostate cancers will progress into an androgen-independent stage. The detailed molecular mechanism of this phenomenon, the so-called flutamide withdrawal syndrome, remains unclear. Since ARA70 can

enhance the androgenic activity of HF (31,32), we were interested in knowing the effect of various AR coactivators on the enhancement of agonist activity of HF. As shown in Fig. 4, ARA70 and ARA55 could enhance the HF-mediated AR transactivation three- to four-fold (lane 1 vs 2 and 3) with HF treatment. By contrast, other AR coactivators, such as ARA54, SRC-1, and Rb, could only show marginal effects in the presence of 1 μ M HF. Together, these data indicated that some selective AR coactivators can promote the HF-AR-mediated transactivation at pharmacological concentrations, which may provide one explanation why HF has partial agonist activity during androgen ablation therapy of prostate cancer.

Adiol-Mediated AR Transactivation in Presence of Different AR Coactivators

Adiol, derived from dehydroepiandrosterone and convertible into testosterone (34), has been suggested to play a role in the regulation of immune response (35), obesity (36), and genesis of estrogen-sensitive carcinomas, such as breast cancer (37,38). In addition, Adiol has been shown to have estrogenic activity at the physiological concentration (34,39). Because of the Adiol-mediated estrogenic effect on estrogen receptor (ER), Adiol has also been proposed as an essential female hormone to replace E_2 for postmenopausal women. However, the androgenic activity of Adiol itself has been explored only recently, and the evidence indicates that this is an AR-mediated effect and that ARA70 can further enhance Adiol-mediated activation of the AR (33). We were interested in determining whether other AR coactivators could also promote the androgenic activity of this adrenal androgen. As shown in Fig. 4, ARA70, ARA55, and ARA54 could all enhance Adiol-mediated AR transactivation four- to five-fold, whereas the AR coactivators SRC-1 and Rb had only a marginal effect (Fig. 4, lanes 5 and 6) with the 10 nM Adiol treatment.

Specificity of Coactivators for AR, ER, Glucocorticoid Receptor and Progesterone Receptor

To study the specificity of AR coactivators to various steroid receptors, the AR coactivators (ARA70, ARA55, ARA54, SRC-1, and Rb) and classic steroid receptors (AR, ER, glucocorticoid receptor [GR], and progesterone receptor [PR]) were inserted into pSG5 expression vectors for the same transfection efficiency. At a 1:3 receptor:coactivator ratio in the presence of 10 nM of each receptor ligand, our results (Fig. 5) showed that DHT-mediated AR transactivation could be enhanced three- to seven-fold by various AR coactivators in the following order: ARA70 = ARA55 > ARA54 > Rb > SRC-1. By contrast, E_2 -mediated ER transactivation could be enhanced by SRC-1 only two- to threefold, but all other AR coactivators had very little or no enhancement effect (Fig. 5; lanes 4 vs 8, 12, 16, 20, and 24). Among these coactivators, our data also showed that ARA55, ARA54, and SRC-1 could enhance progesterone-mediated PR transactivation up to four- to five-fold (lane 3 vs 7, 9, 13,

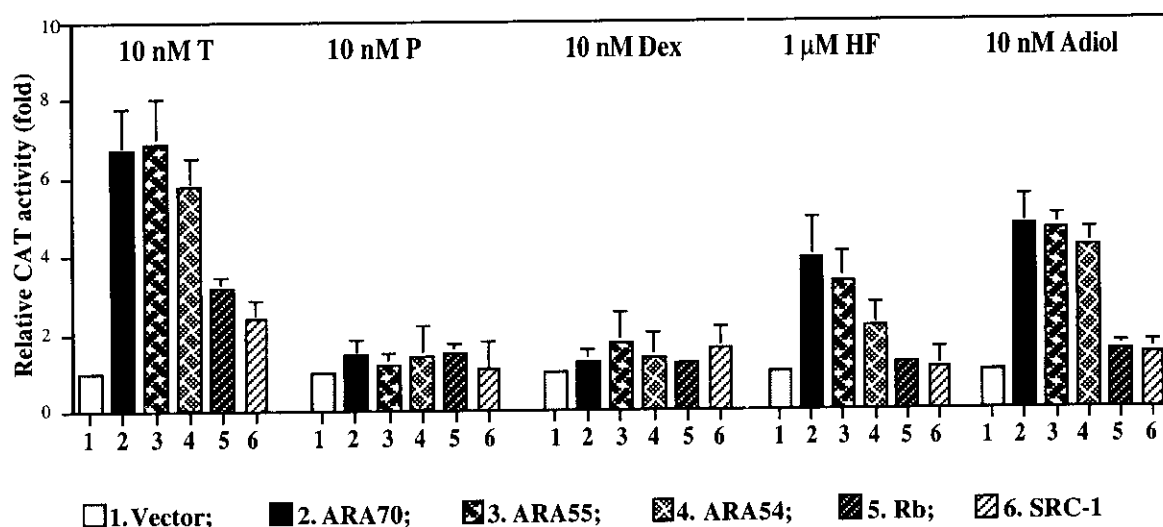


Fig. 4. Characterization of testosterone (T), progesterone (P), dexamethasone (Dex), HF, and Adiol effect on AR-mediated transactivation in the presence or absence of different ARAs in prostate cancer DU145 cells. DU145 cells were cotransfected with wtAR and different coactivators (lanes 2–6). After 24 h of transfection, the cells were treated with 10 nM T, P, Dex, and Adiol, or 1 μ M HF for another 24 h. The first bars show the activity without coactivator (set as onefold). Values represent the mean \pm SD of at least three determinations.

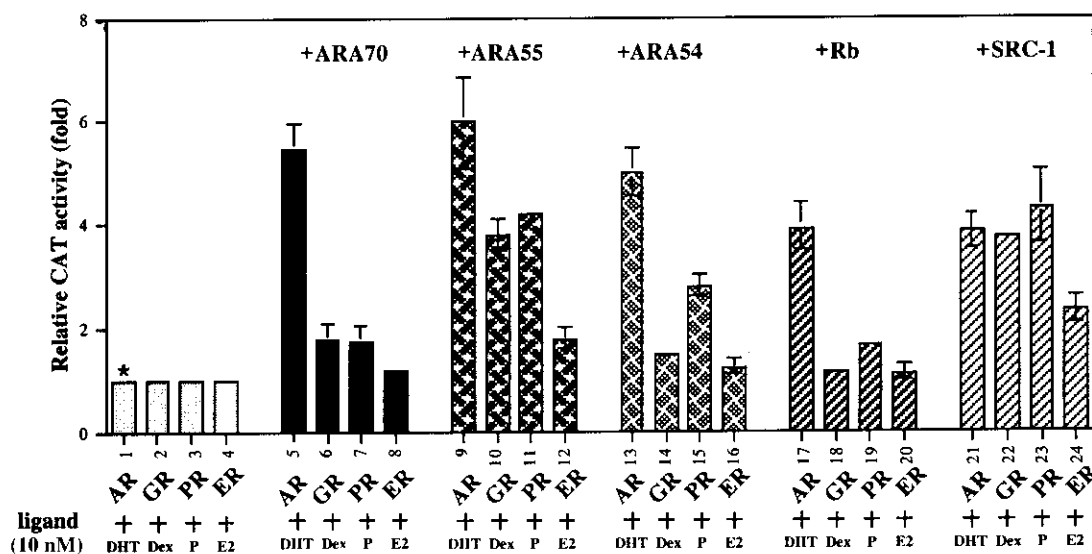


Fig. 5. The effects of different coactivators on the transcription activities of AR, PR, GR, and ER. DU145 cells were transiently cotransfected with 3 μ g of reporter plasmids (MMTV-CAT for AR, PR, and GR; ERE-CAT for ER), 1 μ g of each receptor in pSG5 vector, and 3 μ g of each coactivator. Cells without exogenous AR coactivators were transfected with empty pSG5 vector in the presence of 10 nM of each receptor ligand (*). The CAT activity of each steroid receptor in the absence of coactivator has been standardized to one-fold. The absolute values of induction of each receptor are not identical. Each CAT activity is presented relative to the transcriptional activity observed in the absence of coactivators. Each bar represents the average \pm SD of six independent experiments.

17, and 21) and that only ARA55 and SRC-1 could enhance dexamethasone-mediated GR transactivation up to four-fold (lane 2 vs 6, 10, 14, 18, and 22). In sum, our results suggested that ARA70 has a relatively higher specificity and that SRC-1 can enhance all the steroid receptors.

Interaction of p300/CBP-Associated Factor with AR, ARA70, and ARA54

Recent progress in the study of coactivators has linked the transcriptional activation of steroid receptors to chro-

matin acetylation. Some of these coactivators, such as SRC-1 (40,41) and ACTR/RAC3/P/CIP (42) have been found either to have intrinsic HAT activity or to have the capacity to recruit other factors that have HAT activity (43).

Whereas the histone acetylase, p300/CBP-associated factor (PCAF), has been suggested to be part of a transcriptional complex, the *in vivo* interaction between PCAF and AR, or between PCAF and different ARAs remained unclear. Using the mammalian two-hybrid system, our data (Fig. 6A) indicate that the full-length PCAF can interact

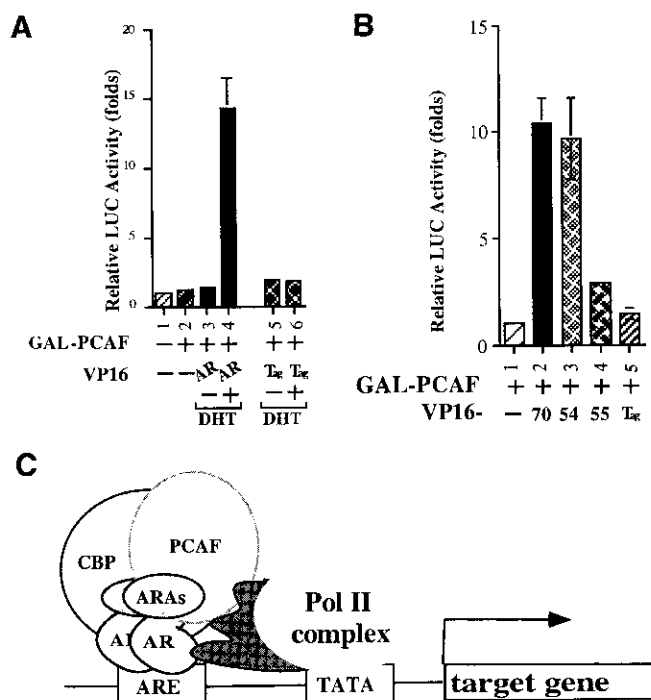


Fig. 6. Analysis of the association of PCAF with AR, ARA70, ARA54, and ARA55 by mammalian two-hybrid assay. (A) The in vivo interaction of PCAF and AR. DU145 cells were transiently cotransfected with 3 μ g of reporter plasmids pG5-LUC, 3 μ g of GAL4DBD fused PCAF (GAL-PCAF), and 3 μ g of VP16-AR fusion. VP-16-fused SV40 large Tag was used as a negative control. One nanomolar DHT was applied on lanes 4 and 6. (B) The interaction between PCAF and ARA70, ARA54, or ARA55. To test the interaction between PCAF and different ARAs, 3 μ g of reporter plasmid pG5-LUC, 3 μ g of GAL-PCAF, and/or 3 μ g of different VP16-ARA fusion were cotransfected into DU145 cells. (C) Schematic presentation of proposed AR-ARAs-PCAF transactivation complex.

with AR only in the presence of DHT (lane 4 vs 3). The SV40 Tag, which could not interact with PCAF, was used as a negative control. We further analyzed the interaction between PCAF and three AR-LBD associated coactivators, ARA70, ARA55, and ARA54. As shown in Fig. 6B, the result indicated clearly that both ARA70 and ARA54 interact well with PCAF (lane 1 vs 2 and 3). By contrast, ARA55 can only interact weakly with PCAF. Thus, in addition to potentiating the AR transactivation, ARA70 and ARA54 could also act as bridge factors to recruit the PCAF in AR transcriptional complex.

With another AR coactivator, CBP/p300 (27), which also has HAT activity, the AR complex may be able to function through these AR coactivators' interaction to modulate chromatin structure for gene transcription (Fig. 6C).

Discussion

Currently, it is well documented that nuclear receptors may need a set of coactivators to modulate their transactivation.

Although SRC-1 has been reported as an effective nuclear receptor coactivator (6), results from transfection assay in DU145 cells indicate that SRC-1 is a relatively weaker AR coactivator (Fig. 1). Previous reports also showed that SRC-1 had only a twofold coactivator effect in CV-1 cells (11). Similar contrasting results also occurred with another coactivator, TIF2, with a 20-fold coactivator effect in COS-7 cells vs only a 4-fold coactivator effect in HeLa cells (44). These contrasting results suggest that the different coactivation effects may be due to different cell environments. Indeed, we also found that ARA70 could become a relatively weaker AR coactivator in other cells, such as PC-3 or HeLa cells (data not shown). It is possible that the different cell environments, the use of various vectors, and the varying ratios of AR to AR coactivators can all influence the coactivator effects. Therefore, it is important to systematically compare the relative enhancement activity of different coactivators under the same conditions.

We have applied different strategies to ensure the androgen-dependent interaction between AR and coactivators. Our results from yeast two-hybrid assay (18,20,21), coimmunoprecipitation (Fig. 2A), and mammalian two-hybrid assay (Fig. 2B) all indicate that the specific interaction between ARAs and AR is an androgen-dependent process. However, using glutathione S-transferase pull-down assay, Alen et al. (44) reported that AR could also interact with ARA70 in the absence of androgen. While the answer to this discrepancy is unclear, the different assay conditions and the existence of other cellular proteins may contribute to these differences. However, the results from our three assays strongly support the notion that the interactions between AR and ARA70, ARA55, and ARA54 are ligand dependent.

To date, prostate cancer has become the most frequently diagnosed neoplasm in the United States and the second leading cause of cancer-related death in American men. So far, the only effective treatment for metastatic prostate cancer is androgen ablation therapy. But the main problem in androgen ablation therapy is that the median duration of response is only 18–36 months. Several studies have attempted to determine how antiandrogens are converted from antagonists to partial agonists following prolonged androgen ablation therapy in prostate cancer patients. One of the alternative explanations might be that the existence of antiandrogens, adrenal androgenic compounds (such as Adiol), or E_2 could still be able to stimulate the AR transactivation. Our data shown here suggesting that some AR coactivators can enhance the androgenic activity of E_2 , HF, or Adiol in human prostate cancer cells may further support this hypothesis and provide some possible explanations for the failure of antiandrogen therapy (Figs. 3 and 4).

In the present study, we are also interested in the specificity of AR coactivators (ARA70, ARA55, ARA54, SRC-1, and Rb) for various steroid receptors (AR, ER, GR, and PR). As shown in Fig. 5, our data suggest that in DU145

cells, ARA70 has the relatively higher specificity for AR, and SRC-1 has the least specificity to AR, with almost equal enhancement with all other steroid receptors. In addition, Rb seems to enhance the AR transactivation but has only marginal effects on other classic steroid receptors. On the other hand, ARA55 was able to enhance most of the classic steroid receptors except ER, and ARA54 could enhance both AR and PR. Because transfection conditions and cell environments may influence the coactivator effects, we could expect that different specificity patterns might occur when we use other cell lines. It therefore will be important to find any factors that can contribute to these contrasting results in different cell environments. Furthermore, because our data only compare the coactivator specificity among classic steroid receptors, the potential coactivator effect on many new members of the nuclear receptor superfamily, such as orphan receptors, remains unclear. Indeed, our data indicate that ARA70 can also function as a coactivator to enhance the transactivation of the peroxisome proliferator-activated receptor in DU145 cells (45). Together, the distinct specificity of each coactivator in different cells may provide cell-specificity for each steroid receptor.

Several lines of evidence indicate that histone acetylation is enhanced in transcriptionally active chromatin (46,47). Coactivators, such as CBP/P300 and PCAF (25,48,49), could further form a large receptor-cofactor complex by binding to other coactivators. The results from our *in vivo* assay indicated that PCAF could interact not only with AR but also with ARA54 and ARA70, providing a linkage from AR and ARAs to histone acetylation that is needed for gene transcription.

In conclusion, some select AR coactivators were able to enhance the androgenic activity of E₂, HF, and Adiol. Additional studies of expression patterns of these AR coactivators in different stages of prostate cancer, both before and after antiandrogen treatment, may further strengthen the roles of these AR coactivators in the progression of prostate cancer from an androgen-dependent to an androgen-independent stage.

Materials and Methods

Materials

DHT and E₂ were obtained from Sigma (St. Louis, MO) and HF was from Schering. pSG5-AR and pSG5-ARA70 were constructed as previously described (18). pSG5-Rb was a gift from W. Kaelin Jr. GAL0, a mammalian expression vector containing the GAL4 DBD, was inserted with the LBD of AR to construct GALAR_{LBD}. pCMX-VP16, containing the activation domain of VP16, was used to construct pCMX-VP16-ARA70, ARA55, and ARA54. pCMX-VP16-AR was constructed by inserting the fragments of AR from amino acid 36 to 918. The sequence of construction junction was verified by sequencing.

Cell Culture and Transfections

Human prostate cancer DU145 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (25 U/mL), streptomycin (25 µg/mL), and 5% fetal calf serum (FCS). Transfections were performed using the calcium phosphate precipitation method, as previously described (18). Briefly, 4 × 10⁵ cells were plated on 60-mm dishes 24 h before transfection with the precipitate containing AR expression plasmid, CAT reporter gene, and coactivator expression plasmid. A β-galactosidase expression plasmid, pCMV-β-gal, was used as an internal control for normalization of transfection efficiency. The total amount of DNA was adjusted to 11 µg using pSG5 in each transfection condition. The medium was changed to DMEM with 5% charcoal-stripped FCS 1 h before transfection. After 24 h of transfection, the medium was changed, and the cells were treated with DHT, antiandrogens, or other treatment. After 24 h, the cells were harvested for CAT assay as previously described (18). The CAT activity was visualized by PhosphorImager (Molecular Dynamics) and quantitated by ImageQuant software (Molecular Dynamics). At least three independent experiments were carried out in each case.

Mammalian Two-Hybrid Assay

Transfections in DU145 cells were performed using the calcium phosphate precipitation method. Briefly, DU145 cells were transiently cotransfected with 3 µg of a GAL4-hybrid expression plasmid, 3 µg of a VP16-hybrid expression plasmid, and 3.5 µg of a reporter plasmid pG5CAT or pG5Luciferase. The cells were then harvested for CAT assays and luciferase assays as previously described (18,50) or according to the manufacturer's protocol (Promega, Madison, WI). A β-galactosidase expression plasmid was used as an internal control for transfection efficiency.

Coimmunoprecipitation of AR and AR Coactivators

Lysates from *in-vitro* translated full-length AR, ARA70, ARA55, and ARA54 were incubated with or without 10⁻⁸ M DHT in modified RIPA buffer (50 mM Tris-HCl; pH 7.4; 150 mM NaCl; 5 mM EDTA; 0.1% NP40; 1 mM phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and pepstatin; 0.25% Na-deoxycholate; 0.25% gelatin). The mixture was rocked at 4°C for 2 h and incubated with rabbit anti-His-tag polyclonal antibodies for another 2 h. Protein A/G PLUS-Agarose beads (Santa Cruz, Santa Cruz, CA) were added and incubated at 4°C for an additional 2 h, then washed four times with RIPA buffer, boiled in SDS sample buffer, analyzed by 8% SDS/PAGE, and visualized by STORM 840 (Molecular Dynamics).

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