

Identification and Characterization of a Novel Androgen Receptor Coregulator ARA267- α in Prostate Cancer Cells*

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The androgen receptor (AR) is a member of the steroid receptor superfamily that binds to the androgen response element to regulate target gene transcription. AR may need to interact with some selected coregulators for maximal or proper androgen function. Here we report the isolation of a new AR coregulator with a calculated molecular mass of 267 kDa named the androgen receptor-associated protein 267- α (ARA267- α). ARA267- α contains 2427 amino acids, including one Su(var)3-9, Enhancer-of-zeste, and Trithorax (SET) domain, two LXXLL motifs, three nuclear translocation signal (NLS) sequences, and four plant homodomain (PHD) finger domains. Northern blot analyses reveal that ARA267- α is expressed predominantly in the lymph node as 13- and 10-kilobase transcripts. HepG2 is the only cell line tested that does not express ARA267- α . Yeast two-hybrid and glutathione *S*-transferase pull-down assays show that both the N and C terminus of ARA267- α interact with the AR DNA- and ligand-binding domains. Unlike other coregulators, such as CBP, which enhance the interaction between the N and C terminus of AR, we found that ARA267- α had little influence on the interaction between the N and C terminus of AR. Luciferase and chloramphenicol acetyltransferase assays show that ARA267- α can enhance AR transactivation in a dihydrotestosterone-dependent manner in PC-3 and H1299 cells. ARA267- α can also enhance AR transactivation with other coregulators, such as ARA24 or PCAF, a histone acetylase, in an additive manner. Together, our data demonstrate that ARA267- α is a new AR coregulator containing the SET domain with an exceptionally large molecular mass that can enhance AR transactivation in prostate cancer cells.

receptor (SR) superfamily that interacts with DNA response elements to regulate target gene transcription (1, 2). AR consists of three main regions: an N-terminal A/B region that contains AF-1, a highly conserved cysteine-rich DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD) (3). The LBD of AR is responsible for ligand binding and contains the interaction surfaces for dimerization.

Recent progress in SR studies indicate that, in addition to contacting the basal transcriptional machinery directly, SRs may inhibit or enhance transcription by recruiting an array of coregulators (4). Several coregulators that are associated with AR have been identified such as ARA70, ARA55, ARA54, ARA24, ARA160, Rb, BRCA1, Smad3, AIB1, and SRC1 (5–14). All of these coregulators can interact with either the C- or N-terminal of AR and enhance AR transactivation (14). While the physiological significance of these identified coregulators remains unclear, several studies suggest some of them may play important roles in SR-related diseases. For example, the overexpression of AIB1 has been linked to the risk of breast and ovarian cancer (15). Variable polyglutamine lengths within AR and AIB1 were also closely linked to the risk of prostate cancer (16),² and ARA24 was associated with the variable polyglutamine lengths in the AR N-terminal domain that may have a role in Kennedy's Neuron disease (8). Furthermore, both ARA55 and Smad3 have been suggested to function as bridges for cross-talk between transforming growth factor- β signaling and androgen/AR action (6, 12). These examples suggest that AR and AR coregulators could play important roles in the development and progression of disease. Here we report the cloning and characterization of ARA267- α , a novel AR-associated protein that contains a Su(var)3-9, Enhancer-of-zeste, and Trithorax (SET) domain.

EXPERIMENTAL PROCEDURES

Materials and Plasmids—5 α -dihydrotestosterone (DHT), dexamethasone, progesterone, 17 β -estradiol (E₂), Δ 5-androstendiol, and dehydroepiandrosterone (DHEA) were obtained from Sigma, and hydroxyflutamide was obtained from Schering. pSG5AR, pSG5ARA55, pSG5ARA54, and pSG5ARA70N (ARA70 N terminus) were constructed as described previously (3, 6, 7, 18). The BRCA1 expression plasmid was

The androgen receptor (AR)¹ is a member of the steroid

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF380302 and AY049721.

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¹ The abbreviations used are: AR, androgen receptor; SR, steroid receptor; DBD, DNA-binding domain; LBD, ligand-binding domain; DHT, 5 α -dihydrotestosterone; E₂, 17 β -estradiol; DHEA, dihydroepiandrosterone; GST, glutathione *S*-transferase; aa, amino acid; TR4, testicular receptor 4; ARA, androgen receptor-associated protein; CAT,

chloramphenicol acetyltransferase; PCAF, P300/CBP-associated factor; MMTV, mouse mammary tumor virus; LUC, luciferase; SET, Su(var)3-9, Enhancer-of-zeste, and Trithorax; PSA, prostate-specific antigen; AR-C, C terminus of AR; AR-N, N terminus of AR; kb, kilobase pairs; NLS, nuclear translocation signals; PHD, plant homodomain; GR, glucocorticoid receptor; PR, progesterone receptor; ER, estrogen receptor; pRL, *Renilla* luciferase reporter plasmid; EST, expressed sequence tag; RACE-PCR, rapid amplification of cDNA ends-polymerase chain reaction; CBP, cAMP-response element-binding protein.

² A. W. Hsing, Y. T. Gao, G. Wu, X. Wang, Y. L. Chen, A. Chokkalingam, J. Deng, J. Cheng, I. A. Sesterhenn, F. K. Mostofi, E. D. Messing, and C. Chang, submitted for publication.

from Michael R. Erdos (Genetics and Molecular Biology Branch, NH-GRI, National Institutes of Health). The Smad3 expression plasmid was provided by Rik Derynck (University of California, San Francisco). We reconstructed the expression plasmid of CBP (provided by Richard H. Goodman, Vollum Institute, Oregon Health Sciences University, Portland, OR) into the pCMV expression vector. pCMX-GAL4AR-C (AR DBD and LBD) and pCMX-VP16AR-N (AR N terminus) were constructed for mammalian two-hybrid assays (11), and pSG5ARA267- α , pGEX-GST-ARA267 α N1, pGEX-GST-ARA267- α N2, and pGEX-GST-ARA267- α C were constructed for the glutathione *S*-transferase (GST) pull-down assay.

Cell Cultures—Human cancer cell lines PC-3, U2OS, SAO2, DU145, HepG₂, and H1299 were grown in Dulbecco's minimal essential medium containing 10% fetal calf serum, penicillin (25 units/ml), and streptomycin (25 μ g/ml). T47D, MCF-7, and LNCaP were maintained in RPMI 1640 with 10% fetal calf serum, penicillin (25 units/ml), and streptomycin (25 μ g/ml).

Yeast Two-hybrid Screening—A MATCHMAKER yeast two-hybrid human brain cDNA library (CLONTECH) that consists of the GAL4 activation domain, amino acids (aa) 768–881, fused with human brain cDNA was used in our yeast two-hybrid screening. The library was screened by co-transformation with a bait construct, GAL4-DBD fused with full-length testicular receptor 4 (TR4) protein, as previously described (5). The transformed yeast Y190 cells were selected for growth on plates with 20 mM 3-aminotriazole and 1 μ M 5 α -DHT but without histidine, leucine, or tryptophan. TR4 is a nuclear orphan receptor with an unknown ligand. Mating tests were used to further confirm protein-protein interaction in yeast cells. One of the initial 31 potentially positive clones reacted firmly with the TR4 and AR-LBD fusion protein (GAL4-DBD-AR-LBD, aa 595–918). This clone was designated Y1600 and selected for further evaluation.

Polymerase Chain Reaction and Cloning of Full-length ARA267- α —Using the sequence of the clone we isolated from the library, we searched the GenBank™ data base. Using the sequence of EST clones, several primers were designed with a 5'-linker containing the restriction enzyme site in order to amplify this clone to full-length. An ~8.0-kb product was amplified, sequenced (BigDye Terminator Kit, PerkinElmer Life Sciences), and subcloned into the pSG5 vector. The polymerase chain reaction template was Marathon human testis cDNA library (CLONTECH) and the program consisted of 94 °C for 1 min, five cycles of 94 °C for 5 s, 72 °C for 12 min, five cycles of 94 °C for 5 s, 70 °C for 12 min, 30 cycles of 94 °C for 5 s, and 68 °C for 12 min. The 5' start codon ATG was confirmed by 5'-RACE-PCR.

Northern Blot and Dot Blot—Human cancer cell lines, PC-3, HepG₂, U2OS, SAO2, T47D, LNCaP, DU145, H1299, and MCF-7 were cultured following the methods previously described. Total RNA was isolated from each cell line using the TRIZOL reagent (Life Technologies, Inc.). 25 μ g of total RNA from each cell line was loaded onto denaturing agarose gels. The RNA samples were separated by electrophoresis and blotted onto a nylon membrane using a vacuum blotter. The Y1600 clone containing a 1.6-kb fragment of ARA267- α (base pairs 911–2542) was used as the hybridization probe, and a β -actin probe was used as a control for equivalent RNA loading. A human multiple tissue RNA dot blot, purchased from CLONTECH (catalog number 7775-1), was also hybridized with the same ARA267- α (Y1600 clone) probe to evaluate tissue distributions of ARA267- α in normal human tissues.

Transfection and Reporter Gene Assay—Human prostate cancer cell lines PC-3 and DU145, lung cancer cell line H1299, and hepatoma cell line HepG2 were grown in Dulbecco's minimal essential medium containing 10% fetal calf serum. For transfection, the cells were plated in 60-mm dishes, and experiments were performed by modified calcium phosphate techniques as previously described (5). After incubation for 24 h, the cells were treated with steroid hormones for another 24 h and then harvested for the chloramphenicol acetyltransferase (CAT) assay. The MMTV-CAT reporter gene was used to measure AR transcriptional activity, and a β -galactosidase expression gene (pCMV- β -Gal) was incorporated into the experiments as an internal control (5). CAT activity was visualized by a PhosphorImager (Molecular Dynamics) and quantitated by IMAGEQUANT software (Molecular Dynamics). For the luciferase (LUC) assay, pG5-LUC, pMMTV-LUC, or estrogen response element-LUC plasmid was used as the reporter gene and SV40-*Renilla* luciferase reporter plasmid (pRL) (Promega) was used as an internal control. The dual-luciferase reporter 1000 assay system (Promega) was employed to measure the LUC activity.

Glutathione *S*-Transferase Pull-down Assay—GST-ARA-267- α N- and C-terminal fusion proteins were expressed in *Escherichia coli*

strain BL21 and purified as described by the manufacturer (Amersham Pharmacia Biotech). The purified fusion proteins were resuspended in 100 μ l of interaction buffer (20 mM HEPES pH 7.9, 150 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.1% (v/v) Nonidet P-40, 0.1% (w/v) bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol) and mixed with 5 μ l of ³⁵S-labeled TNT-expressed AR N-terminal, C-terminal, and full-length proteins (TNT-coupled reticulocyte lysate system, Promega) in the presence or absence of 1 μ M DHT and incubated at 4 °C for 5 h. After several washes with NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 6 mM MgCl₂, 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.5% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 8% glycerol), the bound proteins were separated on an SDS-polyacrylamide gel and visualized by PhosphorImager (Molecular Dynamics).

Mammalian Two-hybrid Assay—For the luciferase assay, 3 μ g of pG5-LUC plasmid was used as the reporter gene and 10 ng of SV40-pRL was used as an internal control. We transfected 4.0 μ g of ARA267 and 2.0 μ g of GAL4-AR-C and VP16-AR-N into PC-3 cells, with or without 1 nM DHT, using the calcium phosphate method. The dual-luciferase reporter 1000 assay system was employed to measure LUC activity.

Western Blot Assay—LNCaP cells were transfected with pSG5ARA267- α and pSG5 vector by SuperFect (Qiagen), respectively. After 2 h of transfection, the medium was changed, cells were cultured for 16 h, and ethanol and 10 nM DHT were applied for another 36 h, respectively. The cells were harvested and lysed following the manufacturer's protocol (Santa Cruz Biotechnology). In each sample, 50 μ g of whole-cell lysis proteins were separated on 10% SDS-polyacrylamide gels. After transferring, the membrane was blotted with polyclonal AR antibody (NH27), PSA antibody (Dako Corporation), and β -actin antibody (Santa Cruz Biotechnology). The bands were developed with an alkaline phosphatase detection kit (Bio-Rad).

RESULTS

Cloning and Sequence of ARA267- α —To further understand the function and mechanism of nuclear receptor action, LBDs of AR and TR4, an orphan receptor, were used as bait to fish out the interacting proteins from the yeast two-hybrid system. ARA267- α , which can interact not only with TR4 but also with AR-LBD in the presence of 1 μ M DHT, was isolated. RACE-PCR technology with the isolated DNA insert as template was used, and several primers were then designed to amplify the full-length human ARA267- α from the Marathon human testis cDNA library. Unexpectedly, the amplified DNA was an exceptionally long insert over 8 kb in size. The longest uninterrupted coding sequence within this 8-kb transcript had 2427 amino acids with a calculated molecular mass of 267 kDa (Fig. 1A). Sequence analysis indicates that ARA267- α is a novel human gene, with no homology to previously identified AR coregulators, such as ARA24, ARA54, ARA55, ARA70, or ARA160. ARA267- α contains several important functional domains (Fig. 1A, boxed or underlined). For example, ARA267- α contains one SET domain (aa 1668–1795), two LXXLL motifs (aa 726–730 and aa 1283–1287), three nuclear translocation signals (NLS) (aa 243–260, aa 888–905, and aa 1202–1219), four plant homodomain (PHD) fingers (aa 1274–1320, aa 1321–1377, aa 1438–1482, and aa 1849–1896), and a proline-rich region. In the four PHD finger regions we also found a cysteine-rich region (aa 1277–1342), a Ring finger (aa 1324–1369), and a zinc finger (aa 1884–1909).

A comparison of ARA267- α to the human EST cDNA sequence available from the GenBank™ data base indicates two separate cDNA sequences that matched ARA267- α . One has a sequence identical to the N terminus of ARA267- α . The other, ARA267- β , has a different N-terminal sequence forming a different 279-amino acid sequence that merges into the 11th amino acid of ARA267- α and forms an uninterrupted coding sequence of 2696 amino acids with a calculated molecular mass of 296 kDa (Fig. 1, B and C). The amino acid sequence analysis shows that ARA267- β has 83% homology with the mouse NSD1 (19), a protein that may function as a coregulator for retinoic acid receptor (RAR) and retinoid X receptor (RXR) (19). It is

A

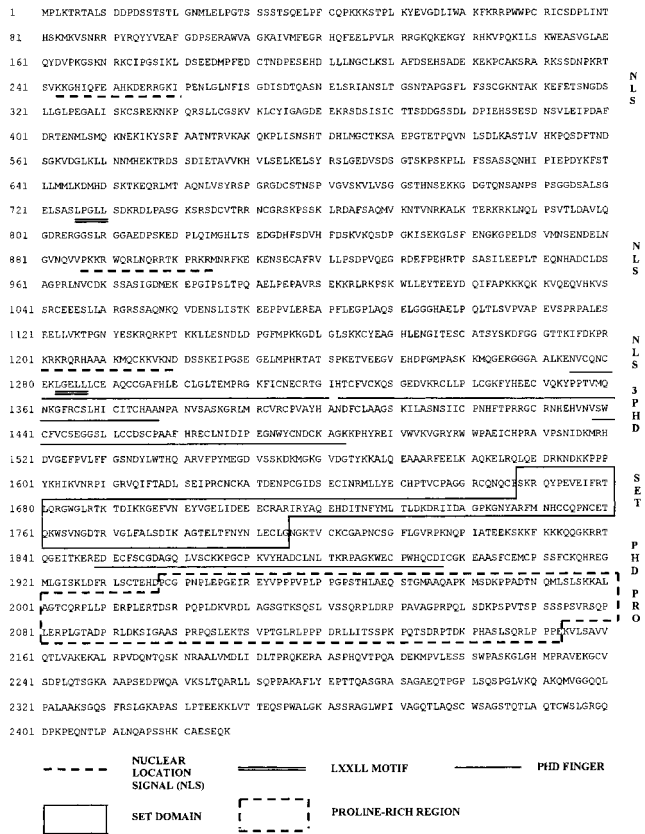
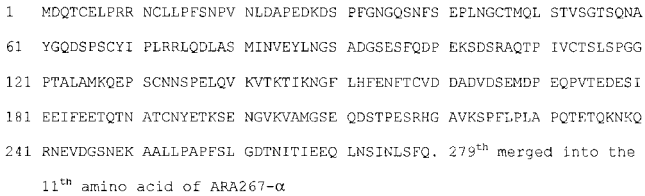
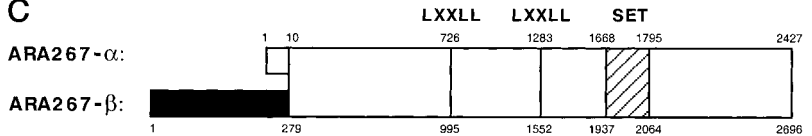


FIG. 1. Amino acid alignment of human ARA267- α . A, the open reading frame of ARA267- α encodes 2427 amino acids. Some potential functional domains are boxed or underlined. Based on data base searches, ARA267- α contains one cysteine-rich region (aa 1277–1342), one SET domain (aa 1668–1795), two LXXLL motifs (aa 726–730 and aa 1283–1287), three nuclear translocation signal (NLS) (aa 243–260, aa 888–905, and aa 1202–1219), and four PHD fingers (aa 1274–1320, aa 1321–1377, aa 1438–1482, and aa 1849–1896) as indicated. B, amino acid sequence of ARA267- β N terminus (residues 1–279), which is different from that of the ARA267- α N terminus (residues 1–10). C, schematic diagram of ARA267- α and ARA267- β indicates the difference in length of the N termini of ARA267- α and ARA267- β .

B



C



possible that ARA267- α and ARA267- β are products of alternative splicing sites although the detailed mechanism remains unclear.

Northern Blot and Tissue Distribution—Northern blot analysis indicates that ARA267- α was expressed as two mRNA transcripts of about 13 and 10 kb in cell lines such as PC-3, U2OS, SAO2, T47D, LNCaP, DU145, H1299, and MCF7 (Fig. 2A, lanes 1–7 and 9) but is absent in the HepG2 cell line (Fig. 2A, lane 8). Multiple-tissue dot blot was used to determine the expression pattern of ARA267- α in different tissues, using prostate as an indicator. We found lung, placenta, uterus, kidney, thymus, lymph node, liver, pancreas, and thyroid gland tissues have higher expression levels than prostate tissue, with lymph node as the highest. In contrast, tissues like bladder, testis, ovary, skeletal muscle, and mammary gland have relatively lower expression levels than prostate tissue (Fig. 2B).

Interaction between ARA267- α and AR—The GST pull-down assay was applied to confirm and further map the interaction domains between ARA267- α and AR that were shown in the

yeast two-hybrid system. Two ARA267- α N-terminal domains, ARA267- α N1 (aa 1–382) and ARA267- α N2 (aa 1–984), and one C-terminal domain, ARA267- α C (aa 1716–2427) were constructed in a GST fusion vector (Fig. 3A). Each of these *E. coli*-generated GST fusion proteins were then incubated *in vitro* with translated [³⁵S]methionine-labeled AR-N (aa 36–553), AR-C (aa 553–918), or full-length AR (Fig. 3A) for the GST pull-down assay. The results indicate that neither GST-ARA267- α N1 nor GST-ARA267- α N2 can interact with AR-N (Fig. 3B, lanes 3 and 4) but both can interact with AR-C (Fig. 3B, lanes 8–11) and full-length AR in the presence or absence of 1 μ M DHT (Fig. 3B, lanes 15–18). The results shown in Fig. 3C further demonstrate that ARA267- α C can interact with the AR-C peptide and full-length AR in a DHT-enhanced manner (Fig. 3C, lanes 7–8 and 12–13). In contrast, ARA267- α C cannot interact with AR-N (Fig. 3C, lane 3). These data suggest that the AR C-terminal (DBD and LBD domain) but not the N-terminal is responsible for the interaction between AR and ARA267- α .

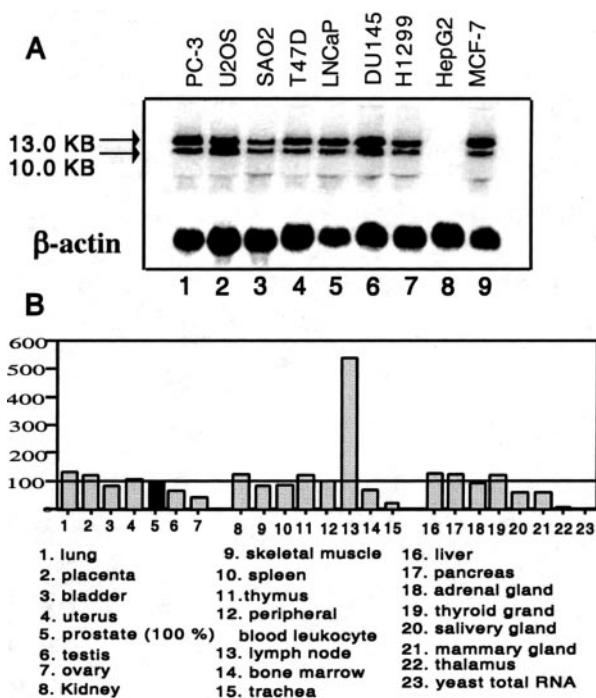


FIG. 2. Tissue distribution of ARA267- α by Northern blot and dot blot. A, Northern blot analysis indicates that ARA267- α is expressed as a mRNA of ~13.0 and 10.0 kb in many cell lines including, PC-3, U2OS, SAO2, T47D, LNCaP, DU145, H1299, and MCF-7 (lanes 1–7 and 9) but is absent in the HepG2 cell line (lane 8). B, multiple tissue dot blots were used to determine the expression of ARA267- α in different tissues, including prostate, testis, adrenal gland, liver, ovary, thymus, etc. The relative expression of ARA267- α was indicated, using prostate as 100%. In lung, placenta, uterus, kidney, thymus, lymph node, liver, pancreas, and thyroid gland tissues (lanes 1, 2, 4, 8, 11, 13, 16, 17, and 19) the ARA267- α expression is greater than 100%, and the remainder are lower than 100% (lanes 3, 6, 7, 9, 10, 12, 14, 15, 18, 20, 21, 22, and 23).

As early data suggested that the N terminus of AR can also interact with the C terminus of AR (36), we were interested in whether the association of ARA267- α with the C terminus of AR can influence the interaction between the N and C terminus of AR. Using the relative LUC activity assay, we found that the coregulator CBP can enhance the interaction between the N and C terminus of AR. In contrast, ARA267- α is more like previously identified coregulators such as ARA70, ARA55, or ARA54 that show little influence on the AR N-C termini interaction (Fig. 4).

Enhancement of AR Transactivation by ARA267- α —Human prostate cancer PC-3 cell line, which is an AR-negative cell line, was transiently transfected with 3 μ g of MMTV-CAT reporter, 1 μ g of AR expression vector (pSG5AR), and with increasing amounts of full-length ARA267- α (pSG5-ARA267- α) in 60-mm culture dishes. The total plasmid amount was adjusted to 11 μ g with pSG5. As shown in Fig. 5A, ARA267- α can enhance DHT-mediated AR transactivation in a dose-dependent manner. Similar results were also observed in human lung cancer H1299 cells (Fig. 5A). To further confirm ARA267- α coregulator activity and rule out the potential artifact effect from the LUC assay, we also performed Western blot analysis to see if ARA267- α can also enhance the AR endogenous target gene, PSA, expression in LNCaP cells. As shown in Fig. 5B, ARA267- α can enhance DHT-induced PSA protein expression. In contrast, ARA267- α showed little induction on AR protein expression.

For the ligand specificity assay, our data show that DHT is the best ligand for the ARA267- α coregulator activity. Unlike ARA70, which was able to enhance AR transactivation in the

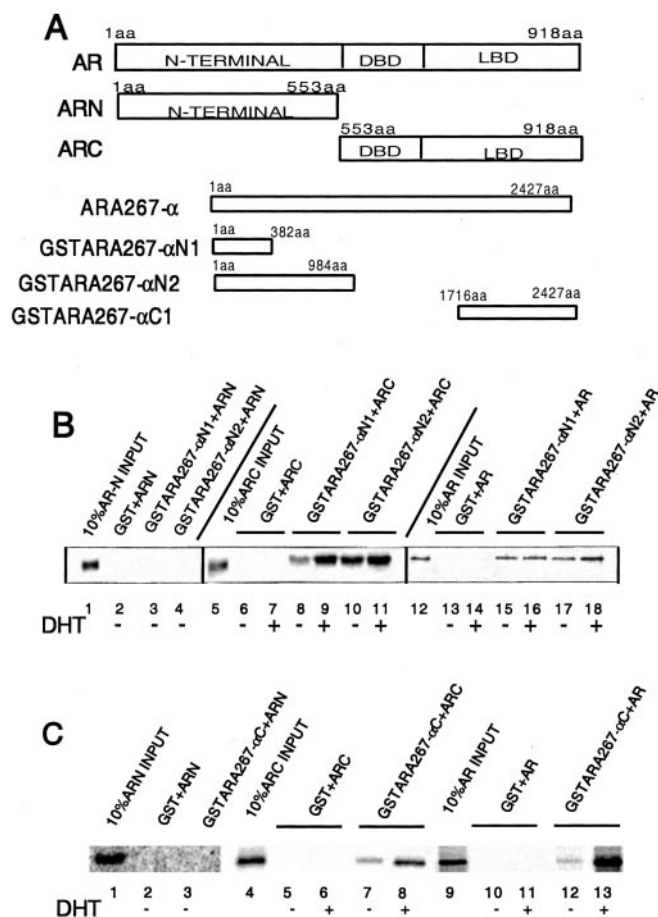


FIG. 3. The interaction between ARA267- α and AR. A, maps of the domains of AR used for ARA267- α interaction and three recombinant GST-ARA267- α fusion proteins, GST-ARA267- α N1, GST-ARA267- α N2, and GST-ARA267- α C. B, all GST fusion proteins were generated in *E. coli* as described. 5 μ l of *in vitro* translated [³⁵S]methionine-labeled AR-N (aa 36–553), AR-C (aa 553–918), and full-length AR were used to perform the GST pull-down assay. 10% TNT expressed AR-N, AR-C, and full-length AR [³⁵S]methionine-labeled products were loaded as controls (lanes 1, 5, and 12). GST was the only control in the absence and presence of DHT (lanes 2, 6, and 13) and (lanes 7 and 14), respectively. Both GST-ARA267- α N1 and GST-ARA267- α N2 cannot pull-down AR-N (lanes 3, 4), but can pull-down AR-C and full-length AR in the presence and absence of 1 μ M DHT (lanes 8–11) and (lanes 15–18), respectively. C, GST-ARA267- α C 10% TNT expression of AR-N, AR-C, and full-length AR [³⁵S]methionine-labeled products were used as controls (lanes 1, 4, and 9). Only GST (also used in lanes 2, 5, 6, 10 and 11) and GST-ARA267- α C cannot pull-down AR-N (lane 3) but can pull-down both AR-C and full-length AR in the presence (lanes 7 and 8) and absence (lanes 12 and 13) of 1 μ M DHT.

presence of other ligands such as E₂, hydroxyflutamide, and Δ 5-androstenediol, ARA267- α shows only marginal effects on AR transactivation in the presence of 10 nM E₂ (Fig. 6).

To test the ARA267- α receptor specificity, we replaced AR with other members of the SR family, such as the GR, PR, and ER, in the LUC assay with HepG2 cells that do not express endogenous ARA267- α . As shown in Fig. 7, ARA267- α has better coregulator activity on AR as compared with PR. In contrast, ARA267- α only has a marginal effect on the transactivation of GR and ER. Similar results also occurred when we replaced HepG2 cells with PC-3 cells.

ARA267- α Additionally Enhances AR Transactivation with Other AR Coregulators—Since it has been demonstrated that several AR coregulators have the capacity to enhance AR transactivation (5–13), we were interested in determining if our newly identified ARA267- α has any additive or synergistic

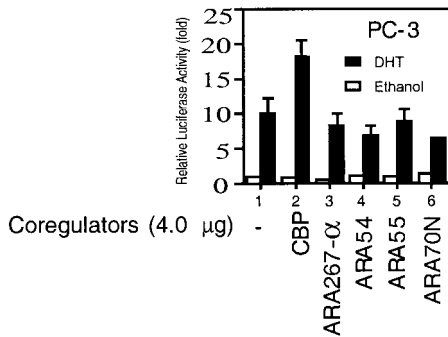


FIG. 4. **ARA267- α does not affect the interaction between the N and C terminus of AR.** PC-3 cells in 60-mm dishes were transiently transfected with 3 μ g of the reporter gene plasmid pG5-LUC, 2 μ g each of GAL4-DBD-fused C terminus AR and VP16-fused N terminus AR and 10 ng SV40-pRL plasmid. Cells were also transfected without or with 4 μ g of pSG5ARA267- α (lanes 1 and 3, respectively) and other AR coregulators in the absence and presence of DHT as indicated. The LUC activity of the interaction between GAL4AR-C and VP16AR-N in the absence of coregulator and DHT was standardized to 1-fold. All values represent the mean \pm S.D. of three independent experiments.

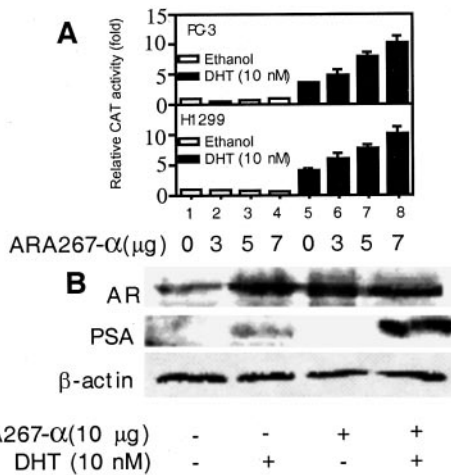


FIG. 5. **The effects of full-length ARA267- α on AR transactivation.** A, PC-3 and H1299 cells in 60-mm dishes were transiently co-transfected with 3 μ g of MMTV-CAT reporter gene, 1 μ g of AR expression vector (pSG5AR), and increasing amounts of full-length ARA267- α as indicated, using the calcium phosphate precipitation method. The total amount of plasmid was adjusted by pSG5 vector to 11 μ g for each transfection. Cells transfected without pSG5-ARA267- α (lanes 1 and 5) and with increasing concentrations of 3, 5, and 7 μ g of pSG5-ARA267- α (lanes 2-4 and 6-8) in the absence (open bars) and presence (closed bars) of DHT indicate that ARA267- α enhanced AR transcription activity in a ligand-dependent manner. The CAT activity without ARA267- α and DHT was set as 1-fold. All values represent the mean \pm S.D. of three independent experiments. B, the endogenous PSA expression was further induced by ARA267- α in the presence of 10 nM DHT. LNCaP cells were transfected with ARA267- α and parental vector as indicated in 10-cm dishes by SuperFect. After 2 h of transfection, the medium was changed for 16 h, and ethanol and 10 nM DHT were applied for another 36 h. In each experiment, 50 μ g of whole-cell extract was applied for Western blotting.

effects with other coregulators on AR transactivation. As shown in Fig. 8, we found that ARA267- α can additionally enhance AR transactivation with other AR coregulators, such as ARA24 (8) or PCAF, a coregulator with histone acetylase activity (14) in PC-3 cells. Together, our data demonstrate that ARA267- α functions as a coregulator to increase AR transcription activity in a ligand-dependent manner.

DISCUSSION

ARA267- α is the first identified AR coregulator that contains the SET domain, an evolutionarily conserved sequence that has

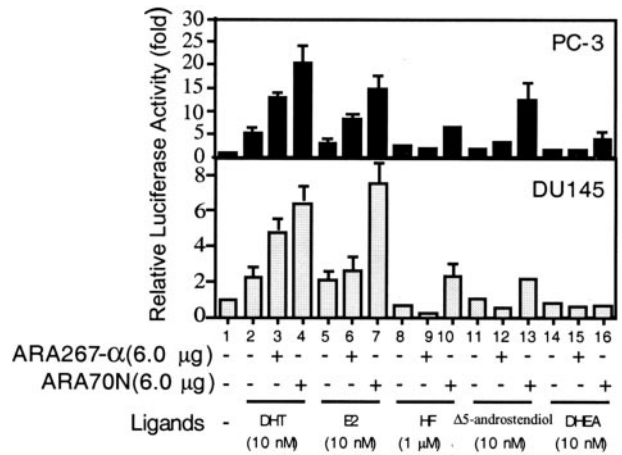


FIG. 6. **ARA267- α effect on AR transactivation with different ligands.** PC-3 and DU145 cells were transiently co-transfected with 3 μ g of MMTV-LUC reporter gene, 1 μ g of pSG5-AR, 6 μ g of ARA267- α , or 6 μ g of ARA70N as indicated and then treated without or with different ligands: 10 nM DHT, E₂, Δ 5-androstendiol, DHEA, or 1 mM hydroxyflutamide (HF). After 24 h, the LUC assay was performed. The luciferase activity of AR without coregulator or ligands was set as 1-fold (first bar). All values represent the mean \pm S.D. of three independent experiments.

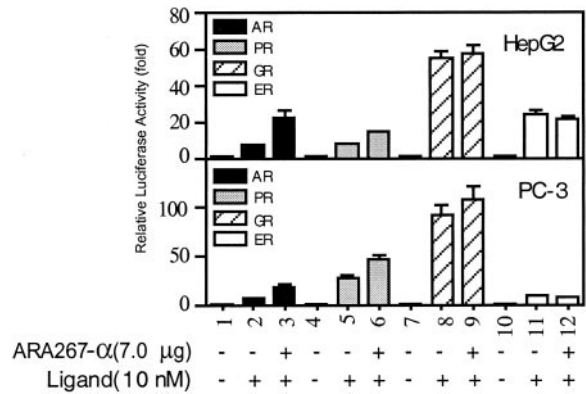


FIG. 7. **Full-length ARA267- α effect on AR and other steroid receptor transcription.** HepG2 cells (an ARA267-negative cell line) and PC-3 cells were co-transfected with 1.0 μ g of various nuclear receptor gene plasmids: 3 μ g of reporter gene plasmids (MMTV-luciferase plasmid for AR, PR, and GR; lanes 1-3, 4-6, and 7-9, respectively) and the estrogen response element-luciferase report plasmid for ER; lanes 10-12; 10 ng of SV40-pRL; and 7 μ g of pSG5-ARA267- α plasmids in the absence and presence of 10⁻⁸ M various ligands: DHT (lanes 1-3), progesterone (lanes 4-6), dexamethasone (lanes 7-9), and 17 β -estradiol (E₂) (lanes 10-12). The luciferase activity of each receptor without ARA267- α and ligands was set as 1-fold. All values represent the mean \pm S.D. of three independent experiments.

a 130-amino acid motif named from three originally identified proteins, Su(var)3-9, Enhancer-of-zeste, and Trithorax (20, 21). Early reports suggest that these three proteins are members of the Polycomb and Trithorax group that play important roles in homeotic gene expression in *Drosophila* (22). Recent evidence indicates that human homologues of these genes, such as ALR, huASH, or ALL-1 (23-25), may also play important roles in the regulation of transcriptional activation or repression via direct modulation of the chromatin structure (22), which may result in cell growth control or disease progression (21, 26, 28). The self-interaction of the SET domains may be one of the mechanisms in integrating the activity of these proteins (27). Whether ARA267- α also plays important roles in AR-mediated gene activation via its SET domain is a very interesting question.

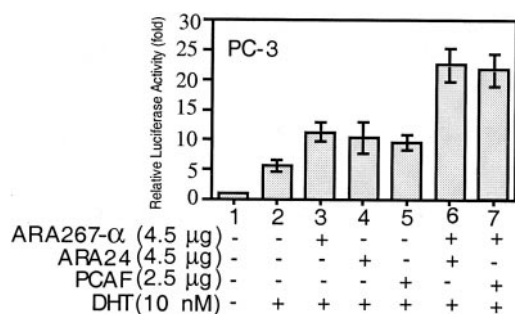


FIG. 8. ARA267- α additionally enhances AR transactivation with other AR coregulators. PC-3 cells were co-transfected with 2 μ g of MMTV-LUC, 10 ng of SV40-pRL, and 0.5 μ g of pSG5-AR as well as ARA267- α , ARA24, and PCAF under various conditions as indicated, in the presence or absence of 10 nM DHT. The LUC activity of AR without ARA267- α and ligand was set as 1-fold. All values represent the mean \pm S.D. of three independent experiments.

One of the most distinct features of SR coregulators is the presence of the LXXLL motif, which plays an important role in interaction between coregulators and receptors for the enhancement of SR transactivation. By mutating LXXLL to LXXAA, Heery *et al.* (29) found that SRC1 failed to function as a steroid receptor coregulator. Similar results also occurred with the TIFII coregulator (30). As ARA267- α contains two LXXLL motifs, we expect these motifs may also play an important role in the enhancement of AR transactivation.

In addition to the SET domain and LXXLL motifs, ARA267- α also contains three nuclear translocation signal (NLS) domains that have been shown to play essential roles for the translocation of proteins from the cytoplasm into the nucleus (31). Furthermore, ARA267- α has four PHD fingers that may play important roles in chromatin-mediated transcriptional regulation. As these PHD fingers overlap with the cysteine-rich region, the zinc finger and the RING finger, we expect that ARA267- α may be able to bind to DNA via these regions. Other proteins with cysteine-rich regions, such as the members of the Trithorax or Polycomb groups, are well known for their roles in chromatin-mediated transcriptional regulation (32). Recent reports link some PHD finger proteins to chromatin remodeling via histone acetylation (33). Other SR coregulators, such as TIF1 α and CBP/p300 also contain PHD finger motifs and have been demonstrated to play important roles in SR-mediated gene transcription. Together, we speculate that based on sequence analysis, ARA267- α may play important roles in AR-mediated gene transcription via the SET domain or PHD fingers.

Previous reports suggest that the interaction between the N and C terminus of AR might play some role in AR-mediated gene transcription (34–36). Some selected AR coregulators, such as CBP, have been shown to facilitate the interaction between the N and C terminus of AR. Since we were unable to show that ARA267- α has any significant influence on this interaction, we anticipate that ARA267- α functions through a different mechanism to enhance AR transactivation.

In our previous reports, we have demonstrated that AR transactivation could be enhanced by 10 nM E₂ in the presence of selected coregulators, such as ARA70 (13). Han *et al.* (37), Weigel and co-workers (38), and Truica *et al.* (17) also reported that E₂ could enhance AR transactivation in the presence of ARA70, SRC1, or β -catenin, respectively. The results shown in Fig. 6 confirmed these studies. ARA70N can enhance AR transactivation in the presence of 10 nM E₂. In contrast ARA267- α has only a marginal effect on the enhancement of AR transactivation in the presence of 10 nM E₂. These data, therefore, suggest that different coregulators may have distinct mecha-

nisms to enhance AR transactivation in the presence of various ligands.

The results shown in Fig. 7 indicate that in the HepG2 and PC-3 cells, ARA267- α only has some marginal enhancement effect on the transactivation of other steroid receptors, such as PR, ER, and GR. Since the maximal function of any given steroid receptor could be related to the combination of the availability of the receptors and their relative abundance compared with many other general transcriptional factors and coregulators (which could differ in various cell lines; Ref. 14), we might expect that in other cells, ARA267- α may have different preferential coactivations and may be able to greatly increase the enhancement of other forms of steroid receptor transactivation.

In conclusion, our data show that ARA267- α acts as an AR coregulator to increase AR transactivation. ARA267- α might exert its AR coactivation through modulating chromatin structure and interacting with other transcription factors. Further investigation is warranted to reveal the detailed functions of ARA267- α on AR and its target organs.

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