

Cloning and Characterization of Human Prostate Coactivator ARA54, a Novel Protein That Associates with the Androgen Receptor*

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Androgen receptor (AR) is a member of the steroid receptor superfamily that may require coactivators for proper or maximal transactivation. Using a yeast two-hybrid screening followed by mammalian cell analyses, we identified a novel ligand-dependent AR-associated protein, ARA54, which consists of 474 amino acids with a molecular mass of 54 kDa. We demonstrated that ARA54 might function as a preferential coactivator for AR-mediated transactivation in human prostate cancer DU145 cells. Interestingly, our data also showed that ARA54 could significantly enhance the transcriptional activity of LNCaP mutant AR (ARt877a) but not wild type AR or another mutant AR (ARe708k) in the presence of 10 nM 17 β -estradiol or 1 μ M hydroxyflutamide. These results imply that both ARA54 and the positions of the AR mutation (877 versus 708) might contribute to the specificity of AR-mediated transactivation. Our findings further demonstrated that the C-terminal domain of ARA54 can serve as a dominant negative inhibitor and exogenous full-length ARA54 can reverse this squelching effect on AR transcriptional activity. Co-expression of ARA54 with other AR coactivators, such as ARA70 or SRC-1, showed additive stimulation of AR-mediated transactivation, which indicates that these cofactors may function individually as AR coactivators to induce AR target gene expression. Through our findings, we have identified and characterized a novel AR coactivator, ARA54, which may play an important role in the AR signaling pathway in human prostate.

cently, it has been reported that several nuclear receptors can interact directly with components of the basal transcription machinery apparatus, such as TBP (6), TFIIB (7), and TFIIF (8). In addition, specific sets of proteins were recruited by the steroid receptors as coactivators that may function as bridge factors between the receptors and general transcription factors in the preinitiation complex (9–11).

Identifying and understanding the function of individual components of these complexes are part of the key to answer how nuclear receptors regulate their target genes. Indeed, several cofactors, such as ARA70 (12), SRC-1 (13), CBP/p300 (14), GRIP1/TIF2 (15, 16), Rb tumor suppressor (17), and RAC3/ACTR (18, 19), have been identified as being able to modulate the transactivation of the steroid receptors. More significantly, recent progress in the study of coactivators further linked the transcriptional activation of steroid receptors to chromatin acetylation. Some of these coactivators, such as RAC3/ACTR (19), CBP/p300 (20), and SRC-1 (21), have been found to either have intrinsic histone acetyltransferase activity or have the capacity to recruit the p300/CBP-associated factor (pCAF) that has histone acetyltransferase activity. However, the physiological significance of these cofactors and their involvement in development, differentiation, and reproductive disease remains to be further studied.

Prostate cancer is the most common malignant neoplasm in aging males in the United States. Surgical or chemical castration in combination with antiandrogens, such as hydroxyflutamide (HF), has been widely used for the treatment of this disease (22). However, most prostate cancers undergoing androgen ablation treatment progress from an androgen-dependent to an androgen-independent state (23). Indeed, previous studies using transient transfection assays also showed that antiandrogens might have agonist activity to stimulate mutant AR-mediated transcription (24, 25). It has been hypothesized that cellular factors and mutant ARs might contribute to the alterations of the antiandrogen specificity and the development of resistance to androgen ablation therapy in prostate cancer (26, 27). Therefore, it will be an important issue to investigate whether AR cofactors are involved in the progression of androgen resistance.

Here we report a new AR-associated protein, ARA54, which was isolated from human prostate cDNA library by using a mutant AR, ARt877s (codon 877 threonine to serine), to screen the yeast two-hybrid system. We further provide evidence to demonstrate that ARA54 can function as a coactivator for androgen-dependent transcription on both wild type (wtAR) and mutant AR (mtAR). However, in the presence of 10 nM E2 or 1 μ M HF, ARA54 can only significantly enhance the transcriptional activity of LNCaP ARt877a but not wtAR or mtAR ARe708k (codon 708 glutamic acid to lysine). These results imply that the specificity of antagonist *versus* agonist of anti-

Androgen receptor (AR)¹ is an androgen-dependent transcription factor that belongs to the steroid receptor superfamily (1, 2). Although several studies have revealed how steroid hormone-bound receptors can recognize and interact with hormone-response elements (3–5), the mechanism of how receptors activate their target genes is not fully understood. Re-

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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) AF060544.

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¹ The abbreviations used are: AR, androgen receptor; wtAR, wild type AR; mtAR, mutant AR; ER, estrogen receptor; PR, progesterone receptor; GR, glucocorticoid receptor; ARA, AR-associated protein; DHT, 5 α -dihydrotestosterone; E2, 17 β -estradiol; HF, hydroxyflutamide; DBD, DNA-binding domain; CAT, chloramphenicol acetyltransferase; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction.

androgens could be conferred via the specific configuration between AR structure and different cofactors.

EXPERIMENTAL PROCEDURES

Materials and Plasmids— 5α -Dihydrotestosterone (DHT), dexamethasone, progesterone, and E2 were obtained from Sigma, and HF was from Schering. pSG5 wtAR was used in our previous report (12). pCMV-mtARt877a (mutant AR derived from prostate cancer tumors, codon 877 mutation threonine to alanine) and pSG5mtARe708k (mutant AR derived from a partial androgen insensitive syndrome patient, codon 708 mutation glutamic acid to lysine) were constructed in our laboratory (28).

Screening of Prostate cDNA Library by a Yeast Two-hybrid System and RACE-PCR—A pACT2-prostate cDNA library (a gift from Dr. S. Elledge) that consists of GAL4 activation domain (amino acids 768–881) fused with human prostate cDNA library was transformed into Y190 yeast cells with pAS2-mtARt877s that contains the C-terminal domain of ARt877s (amino acids 595–918) fused with GAL4 DNA-binding domain (DBD) (amino acids 1–147). Transformants were selected for growth on nutrition selection plates with 20 mM 3-aminotriazole and 100 nM DHT without adding histidine, leucine, and tryptophan. Colonies were also filter-assayed for β -galactosidase activity. DNAs from positive clones were recovered from yeast, amplified in *Escherichia coli*, and confirmed by sequencing.

The missing 5' coding region was isolated by 5' RACE-PCR according to the manufacturer's protocol of the Marathon cDNA Amplification kit (CLONTECH). The gene-specific antisense primer used for 5' RACE-PCR was 5'-ttctgtagtttaatttctgaaccttggc-3'. The specific PCR reaction conditions were: 94 °C for 1 min; 5 cycles of 94 °C for 5 s, 72 °C for 3 min; 5 cycles of 94 °C for 5 s, 70 °C for 3 min; and then 25 cycles of 94 °C for 5 s, 68 °C for 3 min. The PCR product was subcloned into pT7-Blue vector (Novagen) and sequenced.

Northern Blot Analysis—The blot (Fig. 2) containing approximately 2 μ g of poly(A)⁺ RNA from human spleen (lane 1), thymus (lane 2), prostate (lane 3), testis (lane 4), uterus (lane 5), small intestine (lane 6), colon (lane 7), and peripheral blood leukocyte (lane 8) was purchased from CLONTECH and hybridized with an ARA54-specific cDNA probe. A β -actin probe was used as a control for equivalent mRNA loading.

Mammalian Two-hybrid Assay—The mammalian two-hybrid system used in our test system mainly followed the CLONTECH protocol, with some modifications. To obtain better expression, the GAL4 DBD (amino acids 1–147) was fused to pSG5, which was driven by the SV40 promoter, and named GAL0. The hinge and ligand-binding domain of wtAR were then inserted into GAL0. Similarly, the VP16 activation domain was fused to pCMX, which was driven by the cytomegalovirus promoter, and named pCMX-VP16 (provided by Dr. R. M. Evans). This pCMX-VP16 was then used to construct full-length fusion of ARA54. The construction junction of each plasmid was verified by sequencing.

Co-immunoprecipitation of AR and ARA54—Lysates from *in vitro* translated ³⁵S-labeled wild type and mutant ARs were incubated with or without 10^{-8} M DHT in the modified RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 0.1% Nonidet P-40; 1 mM phenylmethylsulfonyl fluoride; aprotinin, leupeptin, pepstatin; 0.25% sodium deoxycholate; 0.25% gelatin). The cell-free translated full-length ARA54 fused to S-Tag protein was incubated with S-Tag protein-agarose beads (Novagen) at 4 °C. The conjugated beads were then washed four times with RIPA buffer, boiled in SDS sample buffer, analyzed by 8% SDS-polyacrylamide gel electrophoresis, and visualized by STORM 840 (Molecular Dynamics).

Cell Culture and Transfections—Human prostate cancer DU145 cells and lung carcinoma H1299 cells were maintained in Dulbecco's minimum essential medium containing penicillin (25 units/ml), streptomycin (25 μ g/ml), and 5% fetal calf serum. Transfections were performed using the calcium phosphate precipitation method as described previously (28). Briefly, 3.5×10^5 cells were plated on 60-mm dishes for 24 h, and the medium was changed to Dulbecco's minimum essential medium with 5% charcoal-stripped fetal calf serum 1 h before transfection. A β -galactosidase expression plasmid, pCMV- β -gal, was used as an internal control for transfection efficiency. The total amount of DNA was adjusted to 10.5 μ g with pSG5 or pVP16 in all transfection assays. After 24 h, the medium was changed again, and the cells were treated with various steroids. Cells were harvested after 24 h for chloramphenicol acetyltransferase (CAT) assay as described previously (12). The CAT activity was visualized and quantitated by STORM 840 (Molecular Dynamics). At least three independent experiments were carried out in each case.

RESULTS

Cloning of the Androgen Receptor-associated Protein, ARA54—Estrogen, progesterone, and flutamide were able to activate several AR mutants, including the LNCaP AR mutated in codon 877 (25). The hypothesis that these mutant ARs may change the antiandrogen specificity and contribute to the progress of prostate cancer from an androgen-dependent to an androgen-independent stage has been widely accepted. Therefore, we were interested in investigating whether cofactors are required for mtARs to exert this distinct function. A fusion protein (GAL4-ARt877s) containing the GAL4 DBD and the C terminus of mtARt877s was used as a bait to screen the potential positive clones from a human prostate cDNA library by the yeast two-hybrid system. One of the positive cDNA clones, which can interact with ARt877s, was further isolated and characterized.

Using the 5' RACE-PCR method, we were able to isolate a full-length cDNA sequence (1701 base pairs) that encodes a novel protein with an open reading frame of 474 amino acids (Fig. 1A). The *in vitro* translated product expressed a polypeptide that matched the calculated molecular mass of 53.8 kDa (data not shown), we thus named this AR-associated protein ARA54. The middle portion of ARA54 (amino acids 220–265) contains a cysteine-rich region that may form a zinc finger motif called the RING finger, defined as CX₂CX_{9–27}CXHX₂CX₂CX_{6–17}CX₂C (29) (Fig. 1B). Indeed, several human transcriptional factors or proto-oncogene proteins, including BRCA1 (30), RING1 (31), PML (32), and MEL-18 (33), have been shown to contain this domain. In addition, ARA54 contains a second cysteine-rich motif that has a B-box-like structure located 43 amino acids downstream from the RING finger motif. However, ARA54 does not possess a predicted coiled coil domain immediately C-terminal to the B-box domain (34). This coiled coil domain is highly conserved among the members of the RING finger B-box family; therefore, ARA54 may represent a new subgroup member of this family.

ARA54 mRNA Expression in Different Human Tissues—Northern blot analysis showed that ARA54 mRNA is expressed at the highest level in testis but at a very low level in small intestine and blood leukocyte (Fig. 2). Based on quantitation and normalization to β -actin mRNA using testis as 100%, the expression levels of ARA54 mRNA in the following tissues are: thymus, 17.8%; spleen, 16.6%; colon, 16%; prostate, 14.5%; uterus, 13.2%; small intestine, 5.1%; and blood leukocyte, 3.6%. The ARA54 mRNA also were strongly detected in two other prostate cell lines, PC-3 and LNCaP (data not shown). These observations suggest that ARA54 expression may exhibit a certain degree of tissue or cell-type specificity. The major band that appeared at 3 kilobases was present in spleen, thymus, prostate, and uterus. In addition, there was a second band at 2 kilobases that only appeared in testis. Whether the 2-kilobase mRNA is due to the RNA alternative splice or simply the degraded form of longer mRNA remains to be determined.

Specific Interaction between ARA54 and ARs—To test whether ARA54 could interact with AR in an androgen-dependent manner, we first applied yeast two-hybrid assay. The results showed that DHT and testosterone, at concentrations of greater than 1 nM, promoted the specific interaction between ARA54 and both wtAR and mtAR (data not shown). A mammalian two-hybrid system with CAT reporter gene assay in prostate DU145 cells was further applied to confirm this DHT dose-dependent interaction between wtAR and ARA54 *in vivo*. As shown in Fig. 3A, transient transfection of either ARA54 or wtAR alone showed negligible activity (lanes 2 and 5). However, the CAT activity was significantly induced only when AR was co-expressed with ARA54 in the presence of 10 nM DHT

A

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ggctctctgggtccccctctctgagcactctgaggtcctt
atgtogtcagaagatcgagaagctcaggaggatgaattgctggcc
1 M S S E D R E A Q E D E L L A
ctggcaagatattacgatggagatgaattgagaagacagagctct
16 L A S I Y D G D E F R K A E S
gtccaaggtggagaaccagagatctatttggatttggccacagaat
31 V Q G G E T R I Y L D L P Q N
ttcaagatatttggagcggcaattcaaatgagtgctccagaat
46 F K I F V S G N S N E C L Q N
agtggtcttgaatacaccatttgccttctgctccacttctgctg
61 S G F E Y T I C F L P P L V L
aacttgaactgccaccagattatccactcttccccacttca
76 N F E L P P D Y P S S S P P S
ttcacacttagtggcaaatggctgtcaccactcagctatctgct
91 F T L S G K W L S F P T Q L Q A
ctatgcaagcacttagacaactatgggaagaacacccgtggcagc
106 L C K H L D N L W E E H R G S
gtggtcctgtttgctggatgcaatttcttaaggaagagacccta
121 V V L F A W M Q F L K E E T L
gcatactgaaatattgtctctcttcttggctcaagattgttct
136 A Y L N I V S P F E L K I G S
cagaaaaaagtcagagaagcagctcaagcttctcccaacaca
151 Q K K V Q R R T A Q A S P N T
gagctagatttggaggagctgctgagctgatgtagaccaagag
166 E L D F G G A A G S D V D Q E
gaaattgtgagtagagagcagtgagatggaatcactgca
181 E I V D E R A V Q D V E S L S
aactctgaccagaaattctgactttgatacaagctcagcagata
196 N L L Q E F L D F P Q A Q O I
aaatgcttaataagaaattgttctctgctgctatctcttctgt
211 K C F N S K L F L C S I C F C
gagaagctggtagtgaatgacttcttggatgtagcagc
226 E K L G S E C M Y F L E C R H
gtgactgcaagcctctgagactacttgaatccagatc
241 V Y C K A C L K D Y F E I Q I
agagatggcaggttcaatgctcaactgccagaaacaaagtc
256 R D G Q V Q C L N C P E P K C
ccttctgctgctcctgctcaggtcaagatgtaggaagca
271 P S V A T P G Q V K E L V E A
gagttatttggccttatgaccgcttctctccagctcctctg
286 E L F A R Y D R L L L Q S S L
gacctgatggcagatggtgactgccccggcgtgctgcccag
301 D L M A D V V Y C F R F C C Q
ctgctgtgtgcaaggaactggcaccatgggtatctgctcc
316 L P V M Q E P G C T M G I C S
agctgcaatttgccttctgacttctgctgaggtgacctaccat
331 S C N F A F C T L C R L T Y H
ggggtctccccatgaaagtgactgcagagaatgaatgactta
346 G V S P C K V T A E K L M D L
cgaaatgaatcctgcaagcagatgagcctaataaaagactttg
361 R N E Y L Q A D E A N K R L L
gatcaaggtatggttaagagagtgatcagaagcactggaagag
376 D Q R Y G K R V I Q K A L E E
atggaaagtaaggagtggttagagaagaactcaagagctgccc
391 M E S K E W L E K N S K S C P
tgttgggaactccccatagagaatagacggatgtaacaagatg
406 C S T F I E K L D G C N K M
acatgactgctgtatgcaattctgtggattgcaaggt
421 T C T G C M Q Y F C W T C M G
ctctctctagagcaaaccttacaacatttcaatgacctggt
436 S L S R A N P Y K H F N D P G
tcaccatgttttaaccggctgtttatgctggtggtgtagcagc
451 S P C F N R L F Y A V D V D D
gatatttgggaagatgagtagaagcagtagtaactactgctcaa
466 D I W E D E V E D *
gatatttaactactgctcaagatggaagtggtattgttttccc
taactctcgtcaagtaacaaagtaacttggcggatatttagg
gtactattcattcactctcctgctgagaagatggaagaacga
ggtttatatttcatggtgactactgagaaggtgcatgtgac
attttaaatgtaagtgagaataattataagccaaggttccag
aaaattaaactacagaa

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B

Protein RING Finger domain (CX2CX9-27CXHX2CX6-17CX2C)

ARA54 (220-265) FLCSICFCEKLGSECMYFLCRRHVYCKACLKDYFEIQIRDGQVQCLNC
BRCA1 (22-64) LECPICLELIKE---PVSTRCDHFCKFCMLLKLNLQKKGPSQ---CPLC
PML (55-91) LRCQQCQAEAKC---PKLLPCLHTLCSGCLSEASG-----MQCPIC
RING1 (17-59) LMCPICLDMLKNMTPKE--CLHRFCSDCIVTAL----RSGNKECPTC
MEL-18 (16-56) LMCAICGGYF--IDATTIVECLHSFCKTCI-----VRYLETNKYCPMC

FIG. 1. Nucleotide and amino acid sequence of human ARA54. A, the nucleotide sequence and the deduced amino acid sequence are shown and numbered on the left. Residues constituting the C3HC4 type of the putative RING finger and B-box-like structure are underlined. Sequence data have been deposited into GenBank™ (accession number AF060544). B, alignment of proteins that contain the RING finger motif. Residues conserved in all members are indicated in bold and represent the putative zinc metal ion ligands. X represents any amino acid, and the number of such residues is also indicated.

(lane 3). Our previously identified AR coactivator ARA70 (lane 6) and SV40 large T antigen (lane 7) were used here as positive and negative controls, respectively. Together, these data indicate that the specific interaction between ARA54 and wtAR is an androgen-dependent process in both *in vitro* and *in vivo* assays.

To determine whether the interaction that occurred in yeast or mammalian two-hybrid systems was due to a direct interaction between ARA54 and ARs, co-immunoprecipitation as-

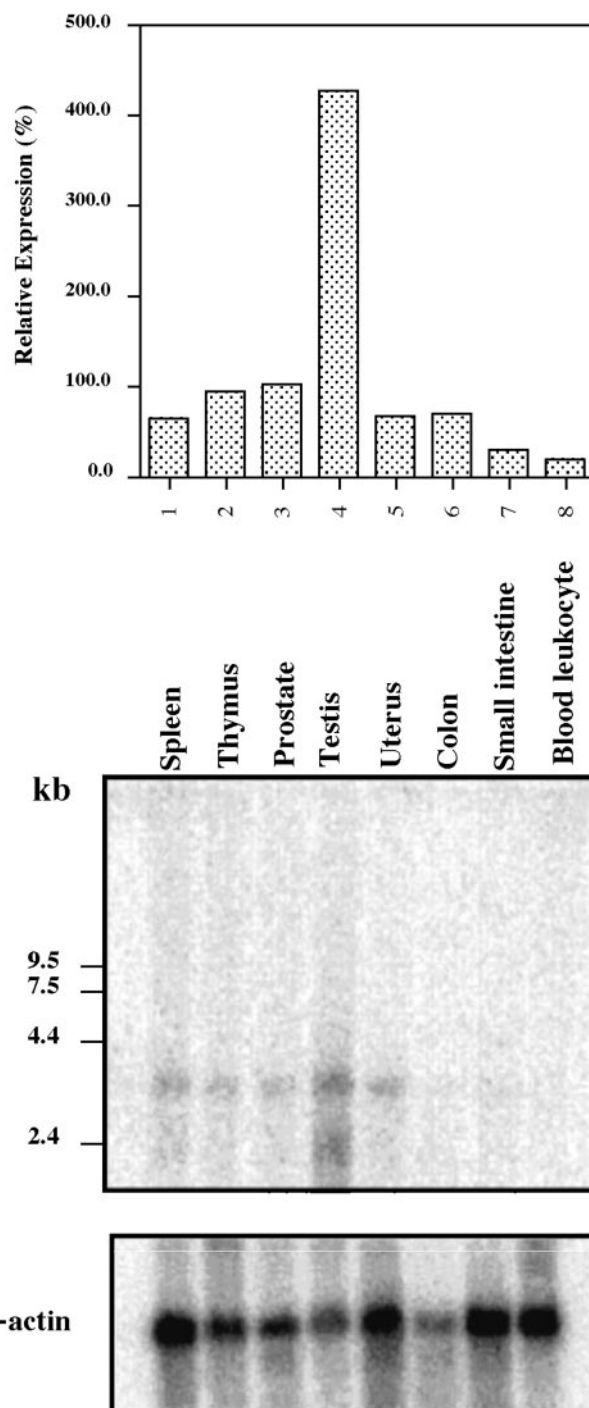


FIG. 2. Northern blot analysis of ARA54 mRNA levels in different human tissues. The blot containing approximately 2 μ g of poly(A)⁺ RNA from human spleen (lane 1), thymus (lane 2), prostate (lane 3), testis (lane 4), uterus (lane 5), small intestine (lane 6), colon (lane 7), and peripheral blood leukocyte (lane 8) was hybridized with an ARA54-specific cDNA probe. A β -actin probe was used as a control for equivalent mRNA loading.

says were performed by an *in vitro* transcription/translation system that expressed various deletion mutants of AR and long-length ARA54 fused to S-Tag protein. The S-Tag-agarose beads used to precipitate the protein complexes were then subjected to SDS-polyacrylamide gel electrophoresis. As shown in Fig. 3B, both the wtAR and LNCaP mtAR could be co-immunoprecipitated by incubation with ARA54 (lanes 1 and 3), and the addition of 10 nM DHT could further enhance this interaction (lane 1 versus lane 2). Moreover, the data showed

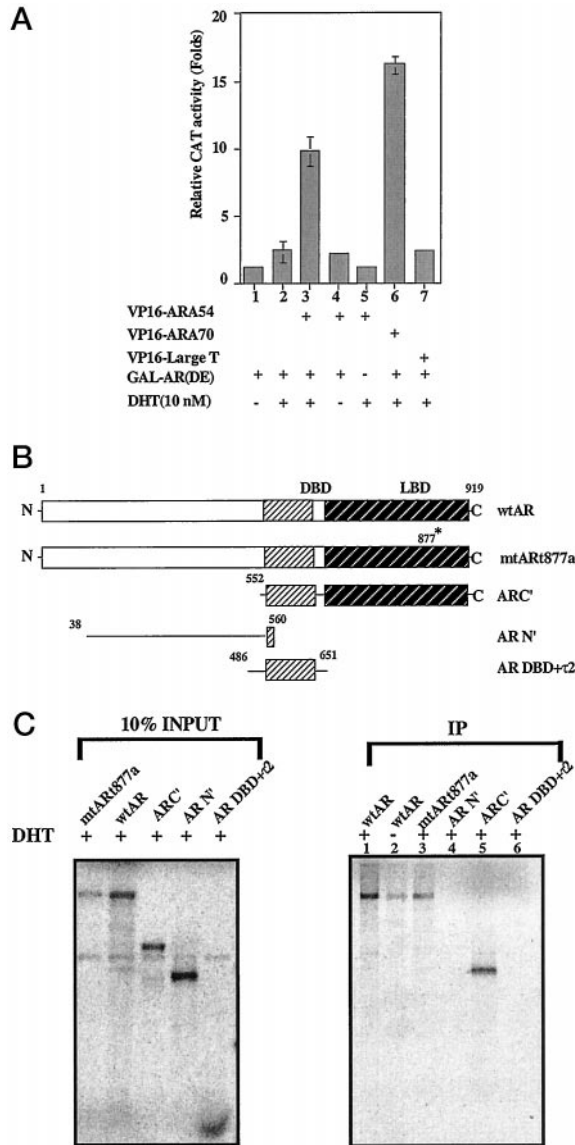


FIG. 3. Specific interaction of ARA54 with AR in DHT-dependent manner. *A*, mammalian two-hybrid assay. DU145 cells were co-transfected with 3 μ g of GAL0 AR encoding the hormone-binding domain of wtAR fused to the GAL4 DBD and 4.5 μ g of VP16-ARA54 encoding full-length ARA54 fused to the activation domain of VP16. Interaction was estimated by determining the level of CAT activity from 3 μ g of the reporter plasmid pG5CAT in the presence of 10^{-8} M DHT. VP16-ARA70 and VP16-SV40 large T antigens were used as a positive and negative control (*lanes 6 and 7*), respectively. *B*, the wtAR and different AR mutants used in the co-immunoprecipitation assay are shown schematically. The DBD and ligand-binding domain (LBD) are indicated. *C*, *in vitro* interactions between AR and ARA54. A series of 35 S-labeled mtARs incubated with ARA54 in the presence or absence of 10 nM DHT are shown in *lanes 1–6*. The S protein-agarose beads were applied to precipitate the protein-antibody complex. 10% of each input protein is shown on the *left panel*. Molecular size markers are in kDa. *IP*, immunoprecipitation.

that the C-terminal region of AR was sufficient to interact with ARA54, whereas neither the N-terminal domain nor DBD plus τ 2 domain of AR (*lane 5 versus lanes 4 and 6*) could interact. Together, these data suggest that the interaction domain within AR could be located between amino acids 652 and 919.

ARA54 Shows Preferential Coactivation with AR and PR—Because several identified cofactors could enhance the transcriptional activity of most steroid hormone receptors (12–17), it is important to investigate whether ARA54 could function as a general coactivator on the transcriptional activity of other

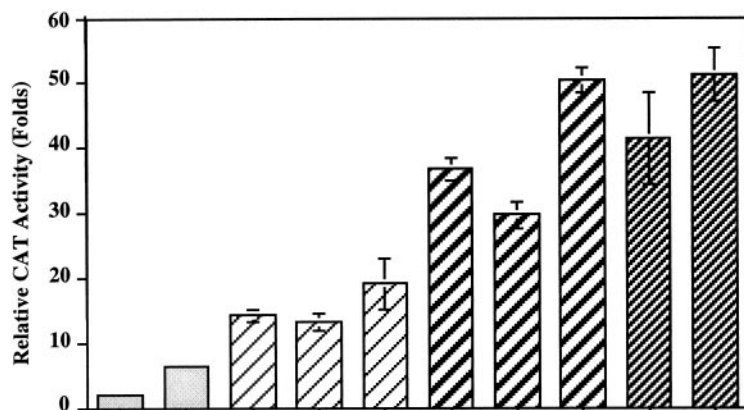
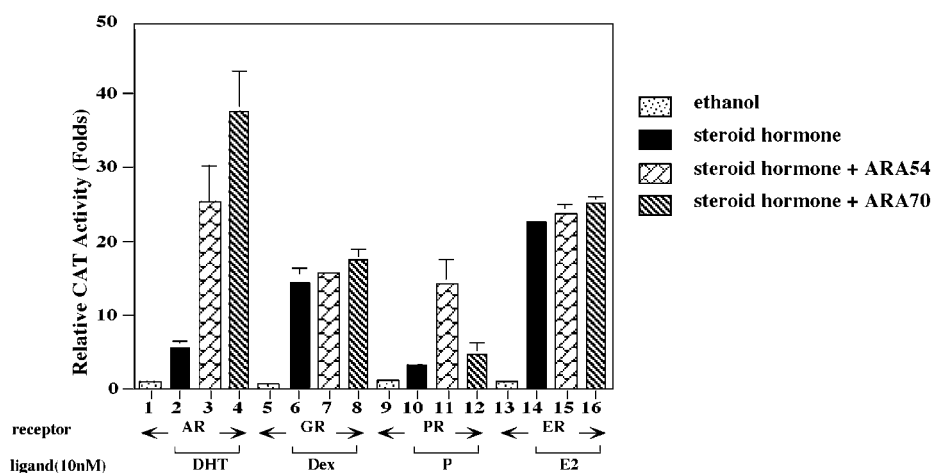
steroid receptors through their cognate ligands and response elements. As shown in Fig. 4, among all the classic steroid receptors we tested, ARA54 could further induce the transcriptional activity of AR and PR up to 6- and 4-fold, respectively. Compared with another AR coactivator, ARA70 that can show higher specificity to AR, ARA54 may represent a less specific coactivator for AR-mediated transactivation. Although ARA54 showed only marginal effects (less than 2-fold) on GR and ER in DU145 cells, we certainly cannot rule out the possibility that ARA54 might be a more general coactivator to other steroid receptors in other cell types under different conditions.

Co-expression of ARA54 with SRC-1 or ARA70 Additively Enhances AR Transcriptional Activity—It has been demonstrated that co-expression of SRC-1 and CBP could stimulate ER and PR transcriptional activity in a synergistic manner (35). In addition, both ARA70 and SRC-1 could act as coactivators for AR transcriptional activation (28). Therefore, we were interested to know if ARA54, together with ARA70 or SRC-1, could synergistically enhance the AR-mediated transcriptional activity. As shown in Fig. 5, ARA54, SRC-1, and ARA70 all induced AR transcriptional activity to 3–5-fold in DU145 cells (*lane 3–5*). Moreover, when ARA54 was co-expressed with SRC-1 or ARA70, an additive, but not synergistic, induction of AR-mediated transactivation was observed (*lane 4 versus lane 9 and lane 5 versus lane 10*). These results indicate that these cofactors may contribute individually to proper or maximal AR-mediated transcriptional activity.

The C-terminal Domain of ARA54 Serves as a Dominant Negative Inhibitor—It has been suggested that the interaction domain of coactivators could interfere with the receptor-mediated target gene expression by squelching the endogenous coactivators present in limited cellular concentration (13, 35). Because the C-terminal region (amino acids 361–474) of ARA54 isolated from prostate cDNA library has been shown to be sufficient to interact with AR in yeast two-hybrid assays, we were interested to test whether it could squelch the effect of endogenous ARA54 on AR-mediated transcription in prostate PC-3 cells. As shown in Fig. 6, the C-terminal region of ARA54 significantly inhibited AR-mediated transactivation (*lane 2 versus lane 6*) and the co-expression of exogenous full-length ARA54 could reverse this squelching effect in a dose-dependent manner (*lanes 7–9*). These results clearly indicate that the C-terminal domain of ARA54 can serve as a dominant negative inhibitor and that ARA54 is required for proper or maximal AR transactivation in human prostate PC-3 cells.

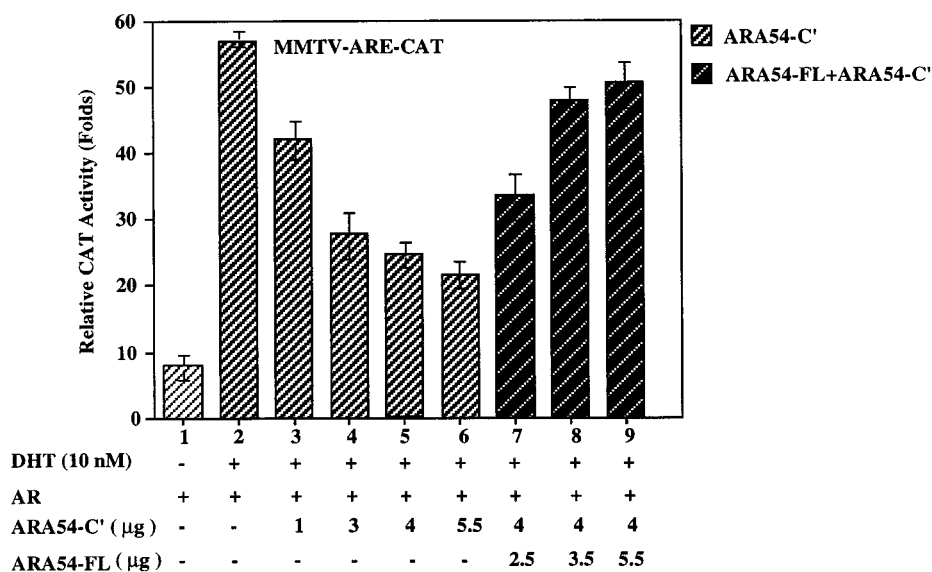
The Effects of ARA54 on the Transcriptional Activities of wtAR and mtARs in the Presence of DHT, E2, and HF—Prostate cancer patients treated with maximal androgen ablation therapy showed undetectable androgens but much higher concentration of antiandrogens (as high as 1–5 μ M) in their serum (36). Thus, it is of interest to us to investigate whether ARA54 can modulate the agonist-antagonist activity of these antiandrogens on wtAR and mtARs. As shown in Fig. 7, wtAR responded well to DHT at 0.1–10 nM, and ARA54 enhanced these transactivations by another 3–5-fold (*lanes 6–8*). However, wtAR responded only marginally to 1–100 nM E2 and 0.1–10 μ M HF in the presence of ARA54 (Fig. 7, *lanes 14–17 and 23–26*). These results are in contrast to the previous identified coactivator, ARA70, that could enhance the wtAR transcriptional activity in the presence of 1 to 10 nM E2 and 1 μ M HF (27, 28, 37). We further extended these findings to two different AR mutants: ARt877a, which was found in many prostate tumors including LNCaP cells (38), and AR ϵ 708k, which was found both in a yeast genetic screening (39) and in one Reifenshtein syndrome patient with partial androgen insensitivity (28). Previous reports showed that LNCaP ARt877s could be stimulated

FIG. 4. Effect of ARA54 and ARA70 on the transcriptional activities of AR, GR, PR, and ER. DU145 cells were transiently co-transfected with 3 μg of reporter plasmids (MMTV-CAT for AR, GR, and PR; ERE-CAT for ER), 1 μg of each receptor constructed in pSG5, and 4.5 μg of pSG5-ARA54-FL (or 3 μg of pSG5-ARA70) in the presence of 10^{-8} M of each cognate ligand. Each CAT activity is presented relative to the transactivation observed in the absence of ARA54, and an error bar represents the mean \pm S.D. of four independent experiments. Dex, dexamethasone; P, progesterone.



DHT (10 nM)	-	+	+	+	+	+	+	+	+
AR	+	+	+	+	+	+	+	+	+
ARA54-FL (μg)			3		6		3	3	
SRC-1 (μg)				3		6		3	
ARA70 (μg)				3		6		3	

FIG. 6. C-terminal domain of ARA54 functions as a dominant negative inhibitor, and full-length ARA54 reverses the squelching effect. Increasing amounts of C-terminal domain (amino acids 361–474) of ARA54 expression vector (pSG5-ARA54C 39) and 3 μg of MMTV-CAT reporter plasmid with 1.5 μg of AR expression vector were co-transfected into H1299 cells in the absence or presence of 10^{-8} M DHT (lanes 1–6). A fixed amount of pSG5-ARA54C 39 (4 μg) was co-transfected with increasing amounts of pSG5-ARA54-FL expression vectors (2.5, 3.5, and 5.5 μg) as described in the text (lanes 7–9). Each CAT activity is presented relative to the transactivation observed in the absence of DHT, and an error bar represents the mean \pm S.D. of four independent experiments.

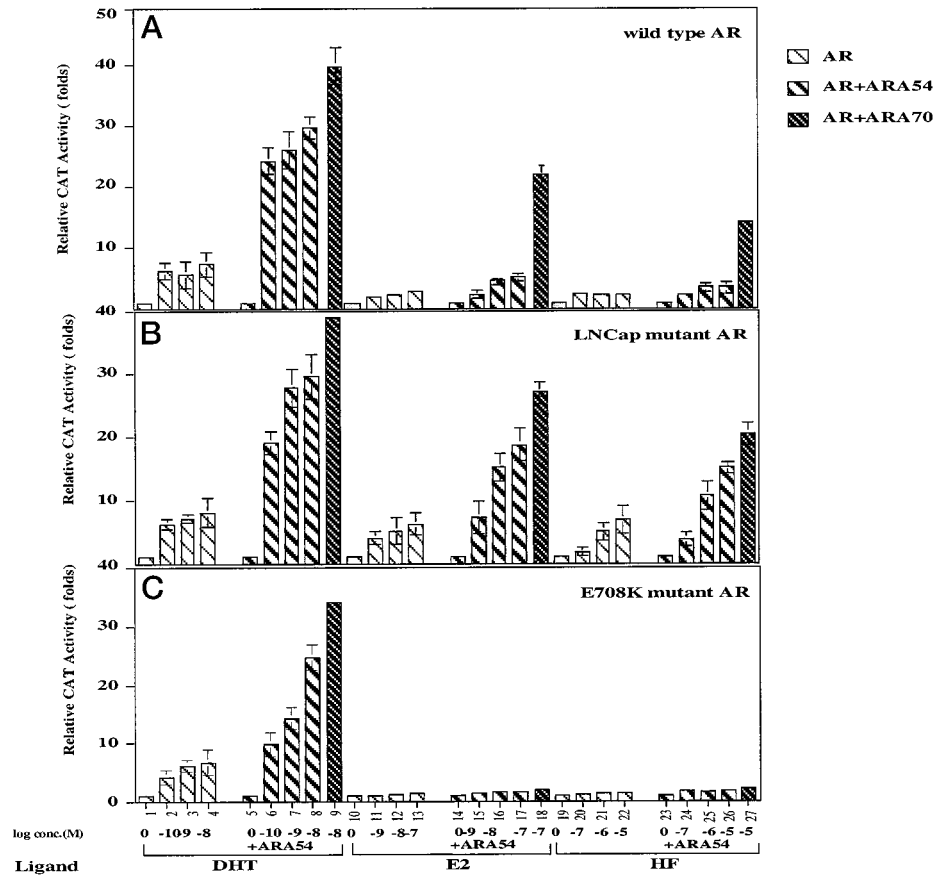


by E2, progesterone, and flutamide (38). In comparison, ARt877a responded to E2 from 1 to 100 nM and to HF from 0.1 to 10 μM . Moreover, ARA54 can further promote this E2- or HF-mediated agonist activity on ARt877a (lanes 10–17). These

results suggest that LNCaP AR might require ARA54 for proper or maximal DHT-, E2-, or HF-mediated transcriptional activity. Unexpectedly, although DHT can still enhance the transcriptional activity of another AR mutant, ARt708k, E2-

FIG. 5. Simultaneous co-expression of ARA54 and SRC-1 or ARA70 enhances AR transcriptional activity additively. DU145 cells were co-transfected with 1 μg of pSG5-AR, 3 μg of MMTV-CAT, and 3 μg or 6 μg of pSG5-ARA54, pSG5-SRC-1, pSG5-ARA70 alone or together with 3 μg of pSG5-SRC-1 or pSG5-ARA70 in the absence or presence of 10^{-8} M DHT (lanes 1–8). Each CAT activity is presented relative to the transactivation observed in the absence of DHT, and an error bar represents the mean \pm S.D. of four independent experiments. Cells in which ARA54, SRC-1, or ARA70 expression vectors were not introduced were transfected with pSG5 parent vector.

FIG. 7. Effect of ARA54 on the transcriptional activities of wild type and mutant ARs. A, 1.5 μ g of wtAR was cotransfected with 4.5 μ g of ARA54 in the absence or presence of DHT, E2, or HF at indicated concentrations. B and C, the LNCaP mtARt877a (B) and mtARe708k (C) were used to replace the wtAR to perform the same experiment as in A. All experiments were performed in DU145 cells, and 3 μ g of MMTV-CAT was used as a reporter plasmid. Each CAT activity is presented relative to the transactivation observed in the absence of ligand, and an error bar represents the mean \pm S.D. of four independent experiments.



and HF-mediated agonist activity on ARE708k, even in the presence of ARA54 (lanes 5–9), is extremely low. Because the codon 708 is located on the helix 3 of the ligand-binding domain and helix 3 has been suggested to play an essential role for the formation of ligand-binding cavity (28), our findings may therefore demonstrate that the residue 708 in helix 3 might be the key component to distinguish the binding between DHT and E2/HF for AR. Together, these results suggest that the AR structure and coactivators, such as ARA54, are both important factors in determining the specificity of sex hormones and antiandrogens.

DISCUSSION

ARA54 described here represents a new AR coactivator with a novel sequence, because neither the nucleotide nor amino acid sequence was found in the EMBL and GenBank™ data bases. Moreover, ARA54 contains a conserved RING finger motif and a B-box-like structure. Proteins in the RING finger family are ubiquitously expressed in species ranging from human to virus, participate in diverse cellular processes, and may be involved in some aspect of transcriptional regulation and protein-protein interaction (40). In addition, it has been reported that mutant PML proteins without the RING finger motif could become the potential dominant negative inhibitors of the wild type PML (41). In agreement with these findings, we have demonstrated that the C-terminal region of ARA54, without the RING finger motif, can serve as a dominant negative inhibitor to attenuate the AR-mediated transactivation. Although the significance of the RING finger domain in ARA54 remains unclear, it is possible that ARA54 might use this domain to interact with other key factors in the activated nuclear receptor-mediated signaling complex and function as a bridge factor between AR and general transcription machinery. Thus, it will be of great interest to further characterize the

functions of the RING finger region of ARA54.

The evidence that the C-terminal domain of ARA54 can function as a dominant negative mutant to inhibit the AR transcriptional activity strongly suggests that ARA54 might play some important roles in the androgen action. Moreover, the incomplete blocking of AR-mediated transcriptional activation by the dominant mutant of ARA54 (Fig. 6) implies that other cofactors like ARA70 and SRC-1 might still function as independent coactivators to induce AR-mediated transcription in the absence of endogenous ARA54. This hypothesis was further supported by the fact that ARA54, ARA70, and SRC-1 could additively induce the AR transactivation. Therefore, each of these cofactors may function via its own individual pathway to promote the AR transcriptional initiation complex for optimal transcription. It will be of particular interest to determine whether AR can simultaneously recruit both ARA54 and SRC-1 or other components of the general transcription factors in the preinitiation complex.

So far, the most popular and effective treatment for advanced prostate cancer is androgen ablation therapy using a combination of surgical or chemical castration and antiandrogens such as HF. Unfortunately, most of the prostate tumors in patients treated with this therapy may relapse within 18 months (13). The mechanism by which prostate cancer cells become resistant to hormone therapy remains unknown. One of the explanations of how prostate cancer progresses from an androgen-dependent to an androgen-independent stage is that mutations in AR may change the specificity and sensitivity of AR to antiandrogens, such as HF (13, 16). It is particularly interesting to investigate whether cofactors may also play important roles in the progression of prostate cancer to an androgen-independent stage. Here we report that in the presence of 10 nM E2 or 1 μ M HF, ARA54 can further enhance the tran-

scriptional activity of LNCaP ARt877a but not wtAR or ARE708k. This suggests that in addition to particular amino acids within the structure of AR, other cofactors such as ARA54 might also contribute to the partial agonist activity of HF and E2 to the mtARs. The differential E2- or HF-mediated AR transcriptional activity between wtAR and mtAR in the presence of ARA54 may provide a nice model for the development of antiandrogens, which will be able to specifically block the mtARs that occur only in prostate tumors.

In summary, our findings suggest that a novel AR-associated protein, ARA54, can function as a relatively specific coactivator for AR in human prostate cancer DU145 cells. Its ability to further enhance E2- or HF-mediated transactivation of ARt887a, but not wtAR, may not only help us to further understand the complexity of the molecular mechanism of androgen action in prostate but also to develop more specific antiandrogens in the battle against prostate cancer.

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