

# Isolation and Characterization of ARA160 as the First Androgen Receptor N-terminal-associated Coactivator in Human Prostate Cells\*

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**The androgen receptor (AR) is a member of the steroid receptor superfamily that may require coactivators for proper or maximal transactivation. Using a purified AR N-terminal peptide as a probe to screen the human testis expression library, we identified an androgen-enhanced AR N-terminal-associated protein ARA160, which consists of 1,093 amino acids with an apparent molecular mass of 160 kDa. Sequence comparison in GenBank™ reveals that ARA160 shares an identical sequence with a HIV-1 TATA element modulatory factor, TMF. The far-Western blotting and co-immunoprecipitation assays demonstrate that the AR can interact directly with ARA160/TMF. Affinity gel pull-down and mammalian two-hybrid assays further suggest androgen can enhance significantly the interaction between AR and ARA160. Transient transfection assays demonstrated that ARA160 might function as a coactivator for AR-mediated transactivation in human prostate cancer PC-3 cells. Our data further suggest that this AR N-terminal coactivator can function cooperatively with AR C-terminal coactivator, ARA70, in PC-3 cells. Together, our data demonstrate that ARA160 might represent the first identified androgen-enhanced N-terminal coactivator for the AR.**

The androgen receptor (AR),<sup>1</sup> like other steroid receptors, is a ligand-inducible transcription regulator that can activate or repress its target genes (1). Deletion analysis reveals that the AR molecule has four major functional domains including the DNA-binding domain (DBD), the ligand-binding domain (LBD), the hinge domain, and the N-terminal activation domain (2, 3). There are two activation functions (AFs) identified in AR: the ligand-independent AF-1 in the N-terminal domain, and the ligand-dependent AF-2 in the LBD. Although AF-1 represents the major transactivation of AR that can be self-transactive in the GAL4DBD (GBD) one-hybrid assays, the androgen may still go through the LBD to control the AF-1

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<sup>1</sup> The abbreviations used are: AR, androgen receptor; GR, glucocorticoid receptor; PR, progesterone receptor; ARA160, AR-associated protein 160; TMF, TATA modulatory factor; DBD, DNA binding domain; LBD, ligand binding domain; AF, activation function; GBD, GAL4 DNA binding domain; DHT, 5 $\alpha$ -dihydrotestosterone; T, testosterone; E<sub>2</sub>, 17 $\beta$ -estradiol; MMTV, mouse mammary tumor virus; LTR, long terminal repeat; PSA, prostate specific antigen; CMV, cytomegalovirus; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

mediated transactivation (4).

Receptor coactivators such as ARA70 (5), ARA55 (6), ARA54 (7), SRC1 (8), TIF1 (9), RIP140 (10), CBP/p300 (11), TIF2 (12), GRIP1 (13), and RAC3/ACTR (14, 15) have been demonstrated to have ligand-dependent association with the LBD of nuclear receptors. Most coactivators have been shown to possess intrinsic activation domains for enhancing receptor transactivity (16, 17). Some coactivators, such as CBP/p300 and SRC1, form a coactivator complex containing histone acetyltransferase activity to modulate chromatin structure, which can influence the accessibility of transcription factors to the chromatin template (18, 19). This suggests a deep impact of steroid hormones on chromatin modulation and transcription regulation.

Here, we report the cloning of ARA160, which has the same sequence as TMF (20), and we demonstrate that ARA160 interacts with the AR in a ligand-enhanced manner. Furthermore, co-transfection of ARA160 markedly enhanced the AR-mediated transcriptional activity on both androgen responsive promoters of prostate specific antigen (PSA) gene and mouse mammary tumor virus long terminal repeat (MMTV-LTR), which suggests that ARA160 is an androgen-enhanced N-terminal coactivator.

## EXPERIMENTAL PROCEDURES

**Peptide Probe Expression and Library Screening**—A human AR (amino acid 38–643) peptide was fused with His-tag and S-tag was expressed in *Escherichia coli* BL21(DE3)pLysS (Novagen, Madison, WI). The S-tagged AR peptide (S-AR 38–643) was purified by immobilized metal affinity chromatography (Amersham Pharmacia Biotech) and was used as a bait in far-Western blotting to screen a human adult testis  $\lambda$ -ZapExpress cDNA library (Stratagene). The expression library was induced by isopropyl-1-thio- $\beta$ -D-galactopyranoside according to the manufacturer's protocol. Eliminating the denaturation and renaturation procedure that was previously used, the membranes were directly blocked with 5% nonfat milk in HBB buffer (20 mM HEPES-KOH, pH 7.5, 100 mM KCl, 5 mM MgCl<sub>2</sub>) at 4 °C for at least 6 h. The membranes were incubated with 0.5  $\mu$ g/ml S-AR 38–643 in blotting buffer (HBB buffer with 1% nonfat milk, 0.02% Nonidet P-40) at 4 °C overnight, and then washed 3 times in washing buffer (HBB buffer with 0.02% Nonidet P-40) at 25 °C for 10 min, followed by incubation with S-protein alkaline phosphatase conjugate in blotting buffer at 25 °C for 30 min. The membranes were extensively washed with washing buffer, and positive clones encoding as S-AR 38–643 associated peptide were further confirmed by far-Western blotting as shown in Fig. 1A.

**Far-Western Blot Analysis**—The ARA160 cDNA (amino acid 410–888) spanning the overlapping region of the three original ARA160 clones was ligated in frame into pET14b and expressed in *E. coli* BL21(DE3)pLysS strain. The bacteria total protein extract lysates were resolved by 7.5% Tricine SDS-polyacrylamide gel electrophoresis, and electrotransferred onto Immobilon-P membrane (Millipore). After five washings, the membrane was probed by another truncated S-tag AR N-terminal peptide (S-AR 38–566), and detected essentially as in the library screening procedure described above.

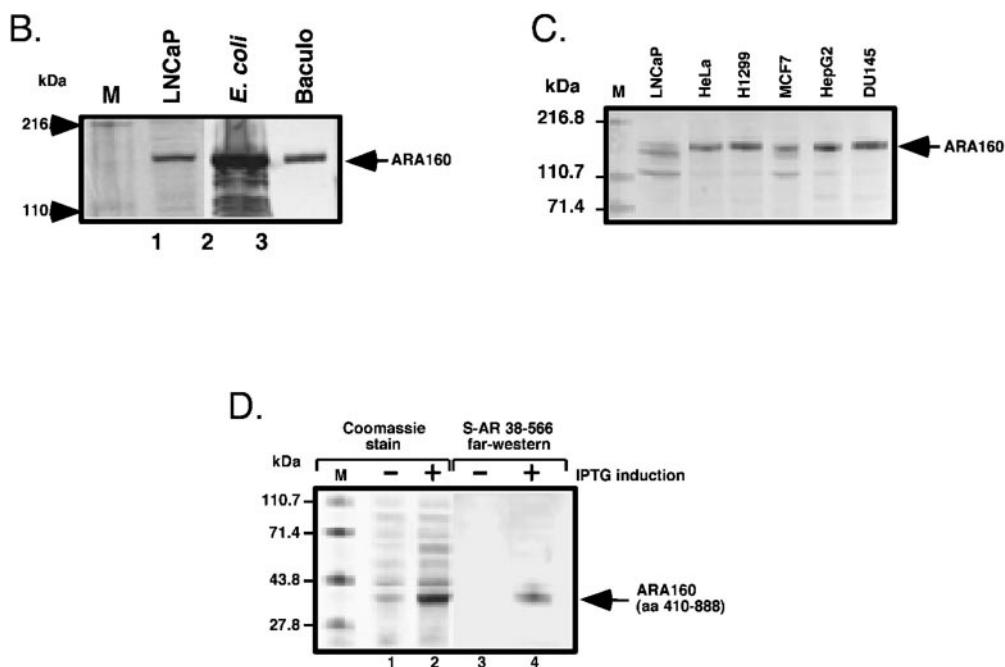
**S-protein Affinity Gel Pull-down Assays**—Full-length ARA160 cDNA was ligated in frame into pET30b (Novagen) to yield S-tag ARA160

## A.

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1  MSWFNASQLS SFAKQALSQA QKSIDRVLDI QEEEPSIWA E TIPYGEFIS SPVSGGWDTS TWGLKSNTPE QSPPIASPKA ITPVRRRTVV DESENFPSAF
101 LSPDTDVQTIQ KSPVVKPPA KSRPEEEVK SSLHESLHIG QSRTPTTES QVKDSSLVCS GETLAAGTSS PKTEGKHEET VNKESDMKVP TVSLKVSSEV
201 IDVKTMTESI SNTSTQSLTA ETKDIALEPK EQKHEDRSN TSPSPVSTPS SGTSTTSDIE VLDHESVISE SSASSRQETT DKSSSLHLMQ TSPQLLSASA
301 CPEYNRLDDF QRLTESCCSS DAFERIDSFS VQSLDSRSVS EINSDDLESG KGYALVPIIV NSSTPKSKTV ESAEGKSEEV NETLVIPTEE AEMEESGRSA
401 TPVNCQPDI LVSSTPINEG QTVLDKVAEQ CEPAESQPEA LSEKEDVCKT VEFLNEKLEK REAQLLSLSE EKALLEEAFD NLKDEMFRVK EESSISLSEK
501 DEPTQRIAEA EKKVQLACKE RDAAKKEIKN IKEELATRLN SSETADLLKE KDEQIRGLME EGEKLSKQQL HNSNIKKLR AKDKENENMV AKLNKKVKEL
601 EELQHLKQV LDGKEEVEKO HRENIKKLS MVERQEKDLG RLQVDMDELE EKNRSIQAL DSAYKELTDL HKANAADSE AQEAALSREM KAKEELSAAL
701 EKAQEEARQQ QETLAIQVGD LRLALQRTSQ AARKEDYLR HEIGELQRL QEAENRNOEL SQSVSSTTRP LLRQIENLQA TLGQSTSSWE KLEKNLSRIL
801 GESOTLLAAA VERERAATEE LLANKIQMSS MESQNSLLRQ ENSRFQALE SEKNRLCKLE DENNRYQVEL ENLKDEYVRT LEETRKEKTL LNSQLEMERM
901 KVEQERKKA I FTQETIKEE RKPFSVSTP TMSRSSISG VDMAGLQTSF LSQDESHDHS FGPMPISAKW KHLYACKDG SRIKHENLQ SQLKLRGEI
1001 THLQLEIGNL EKTRSIMAE LVKLTNQNDE LEEKVKEIPK LRTQLRDLQ RYNTILQMYG EKAEAEELR LDLEDVKNMY KTQIDELLRQ SLS*

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**FIG. 1. Characteristics of ARA160.** A, the amino acid sequence deduced from the full-length open reading frame of ARA160. The AR interacting region of ARA160 representing the overlapping region of the three initial ARA160 clones is *underlined*. B, the endogenous ARA160 from LNCaP nuclear extract and the recombinant ARA160 expressed both in baculovirus and *E. coli* expression systems are shown to have a size of 160 kDa by anti-ARA160 antibody in Western blot. C, ARA160 is ubiquitously expressed in human cell lines at a low level. 100  $\mu$ g of total cellular protein from 6 human cell lines as indicated was analyzed by Western blot with anti-ARA160/TMF antibody. In addition to the common 160-kDa band, 140- and 120-kDa degraded forms were noted in LNCaP and MCF7 samples. D, far-Western blotting was used to confirm the interaction of S-tagged AR N-terminal peptide (S-AR 38–566) with the partial ARA160 (amino acid 410 to 888). Expression of ARA160 (410–888) was induced by isopropyl-1-thio- $\beta$ -D-galactopyranoside as shown in Coomassie Blue staining (*lanes 1 versus 2*). *Lanes 3 and 4* show the specific band recognized by S-AR 38–566 in far-Western blotting.

protein in *E. coli* BL21(DE3)pLysS. S-tag ARA160 was purified by immobilized metal affinity chromatography (Amersham Pharmacia Biotech) and analyzed by Western blot. S-protein-agarose beads were saturated with 1 mg/ml bovine serum albumin, and 5  $\mu$ l of gel for each reaction was loaded with either 500 ng of S-tag ARA160 or 500  $\mu$ g of bovine serum albumin in 100  $\mu$ l of binding buffer (20 mM HEPES-KOH, pH 7.5, 200 mM KCl, 10 mM MgCl<sub>2</sub>, 20% glycerol, 0.1% Nonidet P-40, 5  $\mu$ g/ml leupeptin, 5 mM benzamidine, and 0.1 mg/ml bovine serum albumin) at 4  $^{\circ}$ C with mild agitation for 4 h. After five washes with washing buffer (same as binding buffer without bovine serum albumin), the affinity gels were incubated with 10  $\mu$ l of various [<sup>35</sup>S]methionine-labeled AR proteins (*in vitro* translated by TNT reticulocyte lysate, Promega) in 500  $\mu$ l of binding buffer at 4  $^{\circ}$ C with agitation overnight. After five washes with washing buffer, the beads were resuspended in 30  $\mu$ l of Laemmli sample buffer, boiled for 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Relative band intensity was quantified by PhosphorImager (Molecular Dynamics).

**Plasmids**—pSG5-AR, pSG5-ARA70N, pSG5-AR(E708K) mutant, pCMV-AR(T877A) mutant, pCMX-VP16, and pCMX-VP16AR (38–918) were described previously (21, 22). pCMX-GBDARA160 was constructed by ligating full-length ARA160 in frame to pCMX-gal-N (provided by D. Chen, University of Massachusetts). pCMX-ARA160 was constructed by ligating full-length ARA160 cDNA into pCMX, which is driven by CMV promoter (derived from pCMX-VP16). pG5E1b-Luc and pMMTV-Luc were described previously (23). -1512PSA-Luc was constructed by ligating PSA gene promoter (-1512 to +12) into pGL3.

**Transfection and Reporter Gene Assays**—Human prostate cancer PC-3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were transfected by Superfect transfection reagent (Qiagen) with 2  $\mu$ g of DNA mixture as described previously (23). The same amount of parental expression

plasmid was used as a control and indicated as “–” in the figures. Relative luciferase activities were plotted using the activity of DHT-AR in the absence of coactivator as 1. The results were summarized from at least three sets of transfection and presented as mean  $\pm$  S.E. The results from DU145 cells in Fig. 6, A and B, were transfected by calcium phosphate method as described previously (21).

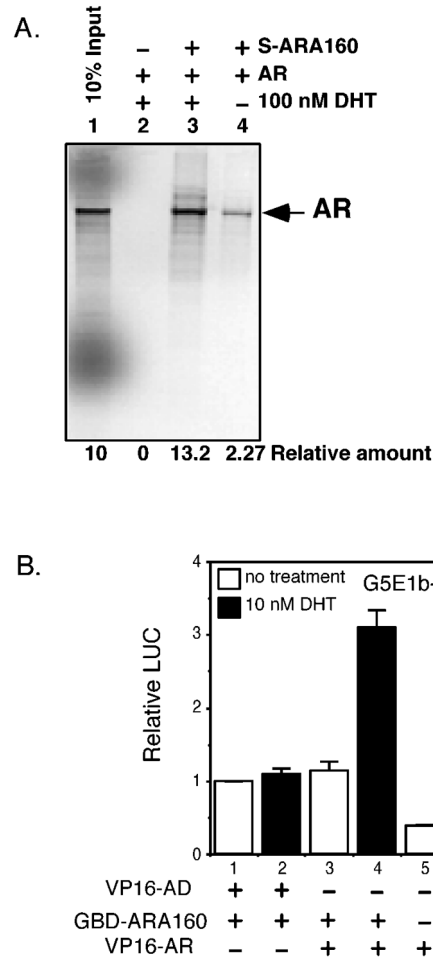
## RESULTS

**Cloning of ARA160 as an AR AF-1 Interacting Protein**—The AR N-terminal domain (AF-1) has been demonstrated as an essential element for AR-mediated transactivation. We were interested to know whether AF-1 needs to interact with an AR cofactor for the proper or maximal AR transactivation. To identify the direct AR AF-1 interacting proteins, an S-tag fused AR AF-1 peptide (S-AR 38–643) was used as a probe to screen human testis cDNA library. From  $6 \times 10^5$  phage plaques, three independent clones corresponding to the same mRNA were isolated from the interaction with the S-AR 38–643. Sequence analysis of these clones revealed a full-length cDNA sequence (3282 base pairs) with an open reading frame of 1093 amino acids (Fig. 1A). The *in vitro* translated product expressed a peptide with an apparent molecular mass of 160 kDa (data not shown). We thus named this AR N-terminal-associated protein as ARA160. Sequence comparison in GenBank™ BLAST search showed that ARA160 has the same sequence as TMF. Neither the AR function nor any promoter other than human immunodeficiency virus-LTR has been reported to be regulated by TMF. Recombinant ARA160 expressed in baculovirus or *E. coli* systems showed the same molecular weight as LNCaP ARA160/TMF (Fig. 1B). Western blot analysis in six human cell lines (LNCaP, DU145, HeLa, H1299, MCF-7, and HepG2) using polyclonal anti-ARA160 antibody that was raised against ARA160 peptide (amino acid 1074–1093) in rabbit, all revealed a ARA160 band (Fig. 1C).

**ARA160 Interacts Directly with AR**—Three different assays (far-Western blotting, co-immunoprecipitation, and affinity gel pull-down) were applied to determine whether ARA160 could interact directly with the AR *in vitro*. With the far-Western blotting, as shown in Fig. 1D, the *E. coli* expressed ARA160 peptide (amino acid 410–888, containing the interacting region based on our initial screening assay) could bind directly to another S-tag fused AR N-terminal peptide (S-AR 38–566). Co-immunoprecipitation assay also confirmed the direct interaction between baculovirus expressed AR and ARA160 with anti-ARA160 antibody (data not shown). Finally, the affinity gel pull-down assay showed that S-tag ARA160 could pull-down [<sup>35</sup>S]methionine-labeled *in vitro* translated AR protein, and this interaction could be significantly enhanced in the presence of 100 nM DHT (Fig. 2A).

Mammalian two-hybrid interaction assays were carried out in human prostate PC-3 cells. When cells were co-transfected with GBD fused ARA160 (GBD-ARA160) and VP16AD fused near full-length AR (amino acid 38–918) (VP16-AR), interaction of these two hybrid proteins formed a functionally transactive complex that activated the transfected GAL4 responsive reporter (pG5E1b-Luc). As shown in Fig. 2B, GBD-ARA160 significantly activated transcription only in the presence of VP16-AR and 10 nM DHT. Together, these mammalian two-hybrid data and the gel pull-down results indicate that androgen enhances AR-ARA160 interaction significantly.

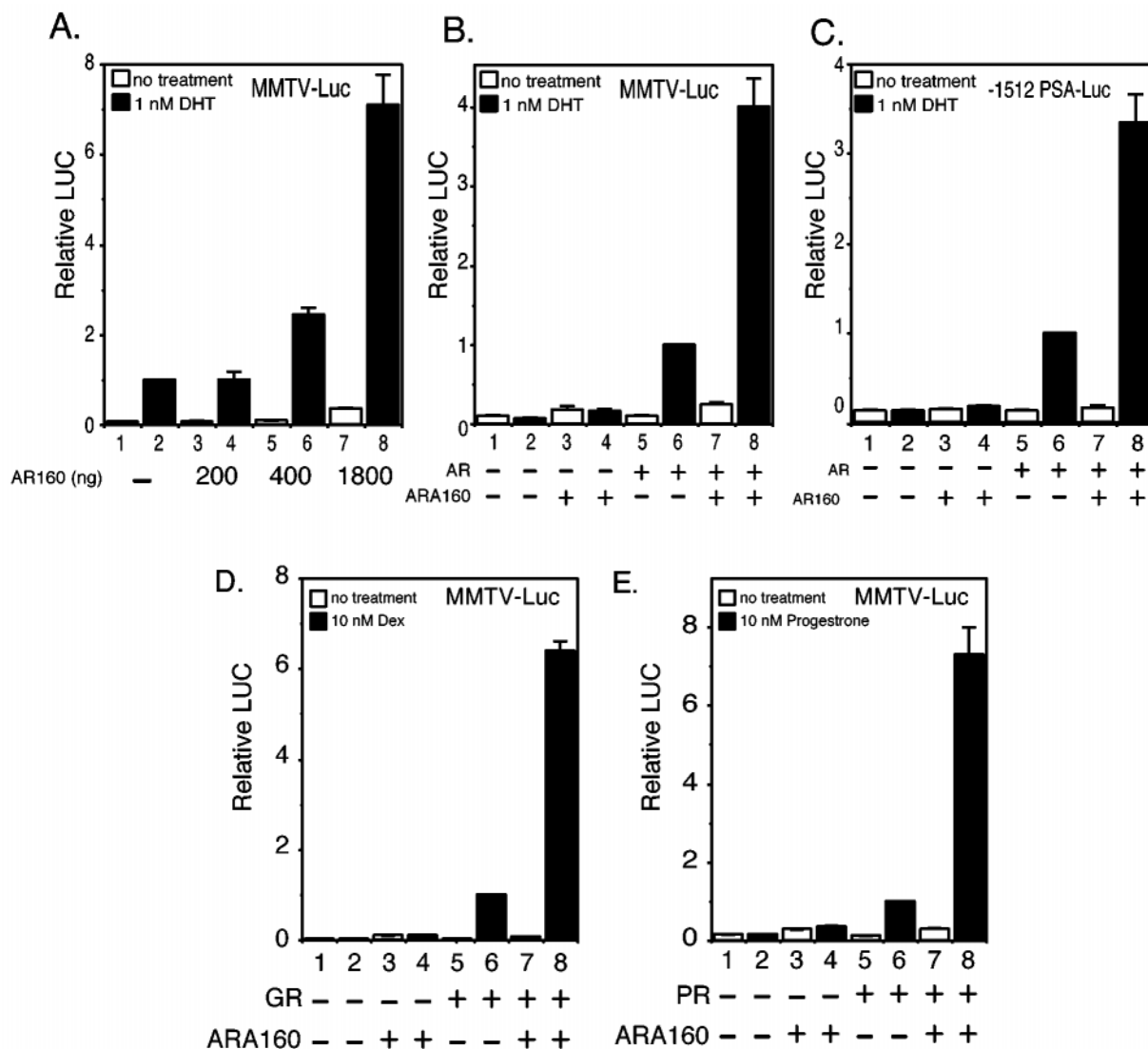
**Influence of ARA160 Expression on the DHT-mediated AR Transactivation**—After demonstrating that AR and ARA160 interaction is enhanced by androgen, we wished to determine whether this interaction could influence the AR-mediated transactivation. As shown in Fig. 3, A and B, co-transfection of ARA160 in increasing amounts enhanced DHT-mediated AR transactivation on MMTV-LTR promoter. When the MMTV-LTR promoter was replaced by PSA promoter, we found that 1



**FIG. 2. Interaction of AR with ARA160.** A, direct protein-protein interaction between AR and ARA160 *in vitro* was analyzed in S-tag gel pull-down assay. S-protein-agarose was preloaded with (lanes 3 and 4) or without (lane 2) S-tag fused ARA160. The affinity gel was then incubated with <sup>35</sup>S-labeled full-length AR in the presence (lane 3) or absence (lane 4) of 100 nM DHT. The relative AR amount retained by each affinity gel was marked at the bottom of the image. B, AR interacts with ARA160 in PC3 cells. The AR-ARA160 interaction was examined by mammalian two-hybrid assays in PC-3 cells. The GAL4 DNA-binding domain fused full-length ARA160 (GBD-ARA160) and the VP16AD fused near full-length AR (amino acid 38–918) (VP16-AR) were transiently expressed in PC-3 cells. Interaction of these two hybrid proteins was examined by determining the level of luciferase activity from the reporter plasmid pG5E1b-Luc.

nM DHT could activate AR transactivation 5-fold (using 1.5 kilobase PSA promoter fused with luciferase as reporter). ARA160 could further induce this AR-induced transactivation up to 17-fold (Fig. 3C). This ARA160 enhanced AR transactivation also occurred when we replaced PC-3 cells with Chinese hamster ovary cells (data not shown). Interestingly, unlike our previous C-terminal coactivator ARA70, which shows high specificity for AR, this N-terminal coactivator ARA160 could also enhance GR- and PR-mediated transactivation using the same MMTV-LTR promoter as reporter (Fig. 3, D and E). These data suggested that ARA160 might represent a more general coactivator to the classic steroid receptors, and ARA160 might interact with some conserved region within AR that shares high homology to the other steroid receptors.

To further analyze the ARA160 interaction to AR AF-1 and AF-2, the N-DBD AR (AR 38–643) and the DBD-LBD AR (AR 553–918) peptides were applied in the affinity pull-down assays. As shown in Fig. 4A, not only N-DBD AR peptide can interact with ARA160 (lanes 1–3), the DBD-LBD AR peptide



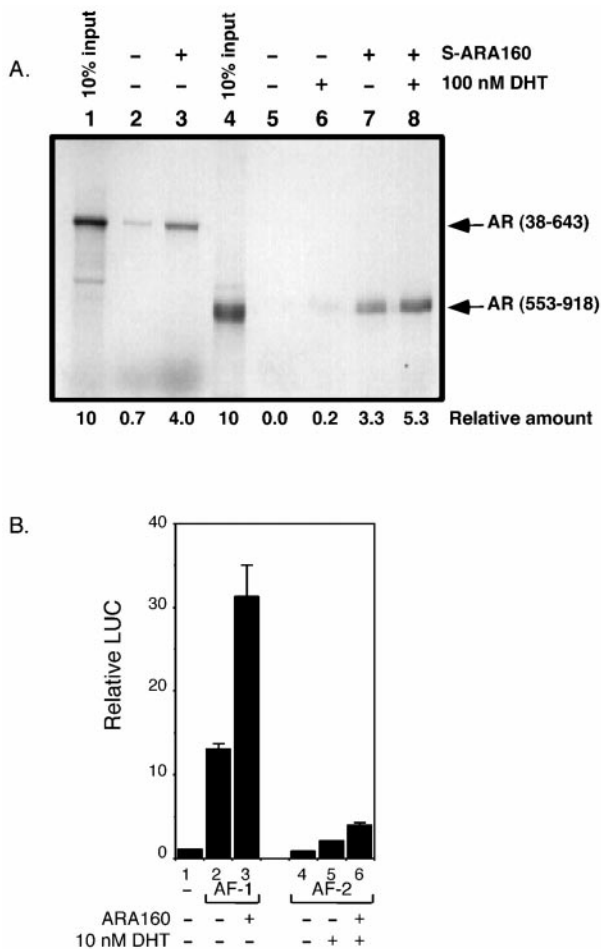
**FIG. 3. Expression of ARA160 enhances AR, GR, and PR transactivation in human prostate PC-3 cells.** A, PC-3 cells were transfected with a DNA mixture containing pMMTV-Luc reporter (100 ng), pRL-CMV (40 ng), pSG5-AR (60 ng), and increasing amounts of pCMX-ARA160. Luciferase activity was measured after another 24 h with (solid bars) or without (open bars) 1 nM DHT treatment. B, transfection was performed as in Fig. 4A with a DNA mixture containing 720 ng of pMMTV-Luc reporter, 40 ng of pRL-CMV, 40 ng of pSG5-AR, and 1200 ng of pCMX-ARA160. C, transfection was performed as in Fig. 4B, except replacing reporter pMMTV-Luc with -1512 PSA-Luc, and pSG5-AR with pCMV-AR. D, transfection was performed as in Fig. 4B, except replacing pSG5-AR with pSG5-GR. E, transfection was performed as in Fig. 4B, except replacing pSG5-AR with pSG5-PR.

can also interact with ARA160 and in a DHT-dependent manner (lanes 4–8). Co-transfection of ARA160 with GBD fused AR AF-1, and AR AF-2 shows that ARA160 enhances the AR AF-1 more than AR AF-2 (Fig. 4B). Notably, the affinity of full-length AR to ARA160 is higher than N-DBD AR or DBD-LBD AR (Fig. 2A versus Fig. 4A), and the ARA160 showed much better enhancement to the full-length AR transactivation, as compared with the AR AF-1 or AR AF-2 transactivation (Fig. 3B versus Fig. 4B). These results indicate that both AF-1 and AF-2 contribute to the AR-ARA160 interaction.

As ARA70 can enhance AR transactivation through the AR LBD (5) and ARA160 can enhance AR transactivation mainly through the AR AF-1 (Fig. 4B), we were interested in determining if any additive or synergistic effects on AR transactivation might occur when both coactivators exist in the same cells. As shown in Fig. 5, whereas ARA160 and ARA70 could enhance AR transactivation up to 6-fold (lane 3 versus 2) and 3-fold (lane 4 versus 2), respectively, co-expression of the AR with ARA160 and ARA70 could further enhance AR transactivation up to 15-fold (lane 5 versus 2). The greater than additive effect sug-

gests that ARA160 and ARA70 can function cooperatively as AR coactivators to enhance AR transactivation.

**Influence of ARA160 Expression on the 17 $\beta$ -Estradiol-mediated AR Transactivation**—As 17 $\beta$ -estradiol ( $E_2$ ) can also induce androgen target genes in the presence of ARA70 (22), we were interested in knowing if ARA160 can also have similar effects. As shown in Fig. 6A, 10 nM  $E_2$  or 1 nM testosterone (T) induced AR-mediated transactivation over 7 and 17-fold, respectively, in the presence of ARA70 in DU145 cells. Under the same conditions, ARA160 had only a marginal enhancing effect on the T- or  $E_2$ -mediated AR transactivation (Fig. 6B). These contrasting results (ARA70 versus ARA160 in DU145 cells, or ARA160 in PC-3 cells versus DU145 cells) strongly suggest that different coactivators might require different conditions for their maximal or proper enhancing effects. Three ARs, the wild type AR, a lose-of-function mutant AR (AR E708K), and a gain-of-function mutant AR (AR T877A), were applied here to test whether mutations within the AR may influence such contrasting enhancing effects between ARA70 and ARA160 (21, 22). As shown in Fig. 6C, whereas 1 nM T and 10 nM  $E_2$

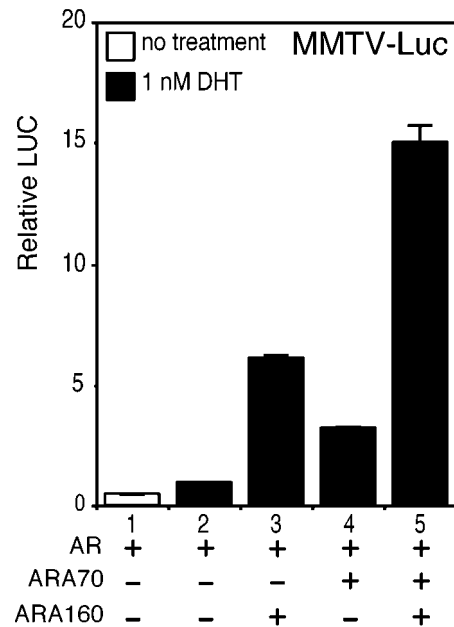


**FIG. 4. ARA160 physically and functionally interacts with AR AF-1 as well as AF-2.** *A*, affinity gel pull-down assays were performed as in Fig. 2*A*, except replacing the full-length AR with N-DBD and DBD-LBD AR peptides (AR 38–643 in lanes 1–3 and AR 553–918 in lanes 5–8). *B*, functional interaction of ARA160 to AR AF-1 (AR 38–566) and AF-2 (AR 668–918). GAL4 DNA-binding domain fused AR AF-1, or AF-2 (300 ng) were transfected in the absence or presence of ARA160 (1200 ng) with pG5E1b-Luc (400 ng), as described in Fig. 2*B*.

could modestly induce the wild type AR-mediated transactivation in PC-3 cells co-transfected with ARA160 (lanes 4 and 6 versus lane 2), 10 nM of T, but not 10 nM E<sub>2</sub>, could induce AR E708K transactivation (Fig. 6*D*, lanes 4 and 6 versus lane 2). In contrast, both 1 nM T and 10 nM E<sub>2</sub> could induce AR T877A transactivation (Fig. 6*E*, lanes 4 and 6 versus lane 2). These data suggest that the cell environment for each coactivator and the amino acid at the position of 708 and 877 may play very important roles for the E<sub>2</sub>-mediated AR transactivation, and that ARA160 has much less effect on E<sub>2</sub>-mediated AR transactivation than ARA70 in DU145 cells.

#### DISCUSSION

Androgens regulate gene transcription through binding to the AR (1, 24). Understanding the transcriptional functions and the regulatory mechanisms of AR is essential for controlling androgen signaling and androgen-regulated prostate cancer growth. Earlier reports suggested that the AR AF-2 may recruit a complex including several AR coactivators, such as ARA70, ARA55, ARA54, and retinal blastoma (5–7, 21), to enhance the AF-2 transactivation function. In contrast, very few coregulators have been reported to selectively bind to the AR AF-1 domain (16), therefore, it will be very important to identify some AF-1 coregulators that can enhance AR AF-1 transactivation.

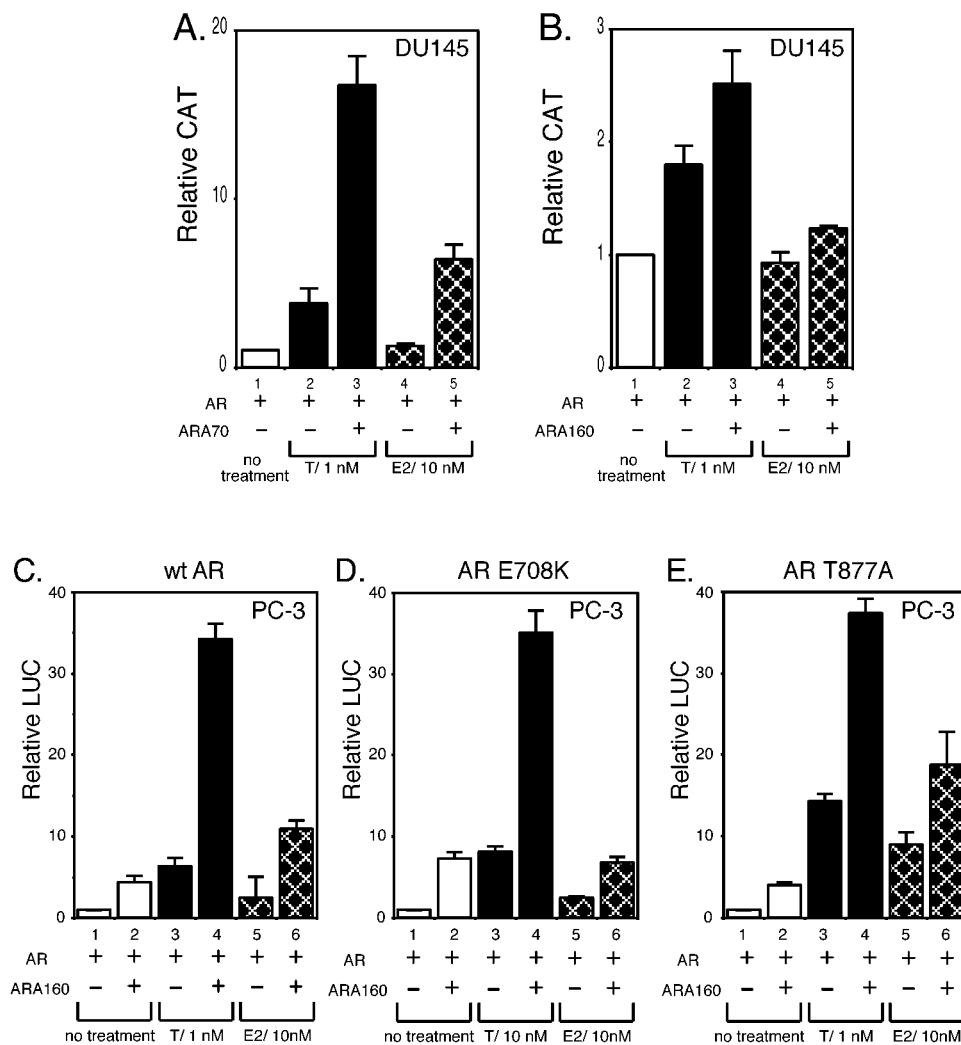


**FIG. 5. Co-transfection of ARA160 and ARA70 synergistically enhances AR-mediated transcription in human prostate PC-3 cells.** PC-3 cells were co-transfected as in Fig. 4*A* with a DNA mixture containing 300 ng of pMMTV-Luc, 40 ng of pRL-CMV, 60 ng of pSG5 AR, 1200 ng of pCMX-ARA160/TMF, and 400 ng of pSG5-ARA70N.

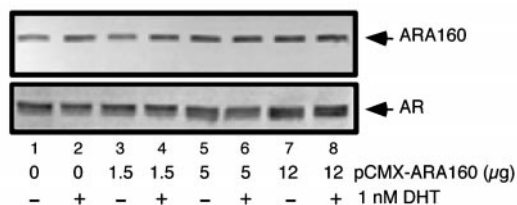
In this study we were able to isolate and demonstrate that ARA160 can function as a coactivator through the interaction with the AR AF-1. Further data indicate that ARA160 can also interact with DBD-LBD of the AR. Although ARA160 enhances the AR AF-1 more significantly than AR AF-2 (Fig. 4*B*), these results suggest that ARA160 may physically and functionally serve as a bridge between AR N- and C-terminal activation domain. Moreover, the greater than additive enhancement of ARA160 and ARA70 on AR transactivation as demonstrated in Fig. 5, further supports the hypothesis that the AR can activate transcription through coordinated mechanisms mediated by both the AF-1 and AF-2. Perhaps, androgen-induced AR can recruit the AF-2 coactivator and the AF-1 coactivator to form a higher AR complex, and cooperation between the AF-1 coactivator and the AF-2 coactivator might expand the androgen-AR signal in transactivation.

Nuclear receptor coactivators, such as SRC1, TRAM-1, and p120, have been reported to show significant coactivator activity only when high doses of coactivator expression plasmids are co-transfected with receptor expression plasmids (25, 26). In the case of SRC-1 and TRAM1, the ratio of coactivator plasmid to receptor plasmid reached 20 to 25:1. This higher transfection ratio of coactivator to receptor also occurred with ARA160, as shown in Fig. 3*A*, more than 10-fold of ARA160 plasmid needs to be co-transfected with AR plasmid for the coactivator enhancing effects. The total ARA160 protein, however, only increases to near 2-fold, even though 30-fold higher ARA160 plasmid than AR plasmid was co-transfected (Fig. 7).

The strong AR coactivation can be only seen in PC-3 and Chinese hamster ovary cells. ARA160 becomes a relatively weak coactivator in DU145, MCF7, and H1299 cells in the same transfection conditions, indicating that intracellular environments, in addition to a high ARA160 plasmid dose, are required for ARA160 to enhance AR transactivation. Compared with ARA70, ARA160 has relative weak coactivator enhancing effects on T- or E<sub>2</sub>-mediated AR transactivation in DU145 cells. However, ARA160 becomes a good coactivator to enhance DHT- and T-mediated transactivation in PC-3 cells. These differen-



**FIG. 6. ARA70, but not ARA160, induces  $E_2$ -mediated AR transactivation in human prostate DU145 cells.** *A*, DU145 cells ( $3 \times 10^5$ ) were transfected with a DNA mixture containing 3.5  $\mu$ g of pMMTV-CAT, 1.0  $\mu$ g of pCMV- $\beta$ -gal, 1.5  $\mu$ g of pSG5-AR, and 4.5  $\mu$ g of pSG5-ARA70N. *B*, DU145 cells were transfected as described in Fig. 6*A*, except replacing pSG5-ARA70N versus pSG5 with pCMX-ARA160 versus pCMX. *C*, PC-3 cells were transfected as described in Fig. 4*B* with 720 ng of pMMTV-Luc, 40 ng of pRL-CMV, 40 ng of pSG5-AR, and 1200 ng of pCMX-ARA160 for 16 h. Transfected cells were treated with hormones (no treatment, 1 nM T, or 10 nM  $E_2$ ) as indicated in the figure for another 24 h. *D*, PC-3 cells were transfected with the same DNA mixture as in Fig. 6*C* except replacing pSG5-AR(E708K) with pSG5-AR(wild type). *E*, PC-3 cells were transfected with the same DNA mixture as in Fig. 6*C* except replacing pCMV-AR(T877A) with pSG5-AR(wild type).



**FIG. 7. ARA160 and AR protein amounts in the transfected cells.** PC-3 cells ( $5 \times 10^6$ ) were transfected with pCMV-AR (0.4  $\mu$ g) and increasing doses of pCMX-ARA160 plasmid as indicated in the figure. Cell lysates were prepared as described in Fig. 1*C*. One-fifth of each lysate was examined by Western blot using anti-ARA160 and anti-AR antibodies.

tial enhancing effects between coactivators support the idea that coactivators require special environments for their maximal or proper function.

Based on the relatively low homology among the N-terminal domain of steroid receptors, we initially expected that ARA160 might function more specifically to AR. However, ARA160 could also enhance GR- and PR-mediated transactivation in transient transfection assays. Possibly, the other portions of

the AR, which have relatively higher homology to GR and PR, contribute to their interaction with ARA160. Nevertheless, our data indicated ARA160 enhanced GAL4-SP1 only marginally (1.5-fold) on pG5E1b-Luc (data not shown), suggesting there are still certain preferences for ARA160 to steroid receptors.

Although the apparent molecular mass of ARA160 (160 kDa) is higher than the calculated molecular mass (123 kDa), the running pattern in SDS-polyacrylamide gel electrophoresis is consistent regardless of expression in either eukaryotic or prokaryotic system. These data may imply the post-translational modification of ARA160 to be not very significant. The fact that ARA160 can function as an AR coactivator in multiple cells, including yeast, PC-3, and Chinese hamster ovary, also supports the above suggestion that ARA160 may function well without special modification. Because the baculovirus expressed ARA160 recombinant protein did not show any band shift with PSA-ARE (androgen response element) and core promoter, and the fact that only minimal self-activation occurred when ARA160 was fused to GBD in transfection assays (data not shown), we might be able to conclude that ARA160 will not bind directly to the core promoter sequence of the AR

target gene and therefore can only function as a coactivator through interaction with the AR in our transfection assays.

In conclusion, we have cloned and identified ARA160 as an AR coactivator through its interaction with the AR N-terminal domain and have shown that ARA160 enhances DHT- and T-mediated AR transactivation in human prostate PC-3 cells. In comparison with ARA70, we found that the cellular environment, ligand, and amino acid within AR are critical for different coactivators to maximally enhance AR transactivation. Further study of the interactions among the AR and all these different AR coactivators will provide us a deeper insight in the regulation of androgen signaling and prostate cancer growth.

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