Androgen receptor (AR) is a hormone-activated transcriptional factor that can bind to androgen response elements and that regulates the transcription of target genes via a mechanism that presumably involves coactivators. We report here the cloning of a novel AR coactivator ARA55 using a yeast two-hybrid system. ARA55 consists of 444 amino acids with the predicted molecular mass of 55 kDa and its sequence shows very high homology to mouse hic5, a TGF-β1-inducible gene. Yeast and mammalian two-hybrid systems and co-immunoprecipitation assays all prove ARA55 can bind to AR in a ligand-dependent manner. Transient transfection assay in prostate cancer DU145 cells further demonstrates that ARA55 can enhance AR transcriptional activity in the presence of 1 nM dihydrotestosterone or its antagonists such as 100 nM dihydrotestosterone or 1 μM hydroxyflutamide. Our data also suggest the C-terminal half of ARA55, which includes three LIM motifs, is sufficient to interact with AR. Northern blot and polymerase chain reaction quantitation showed ARA55 can be expressed differently in normal prostate and prostate tumor cells. Together, our data suggests that ARA55 may play very important roles in the progression of prostate cancer by the modulation of AR transactivation.

The androgen receptor (AR) is a member of the steroid receptor (SR) superfamily and plays an important role in male sexual differentiation and prostate cell proliferation (1). The well conserved DNA binding domain (DBD) within AR has two zinc finger structures that are involved in DNA binding. The C-terminal region of the AR, including the hinge region and the ligand-binding domain, is responsible for the functions of dimerization and androgen binding. The N-terminal region is involved in the transcriptional activation of AR.

The discovery of transcriptional interference/squelching of SRs provided the concept of the existence of transcriptional cofactors that mediate SR function (2, 3). Recently, several putative cofactors (either coactivators or corepressors) for SRs were found and characterized (4, 5). In the studies of the interaction of SRs with these cofactors suggested that these SR-cofactor complexes play essential roles for the regulation of SRs target gene transcription by interaction with general transcription factors and the remodeling of chromatin (4, 5).

The in vivo significance of these cofactors and their relationship to diseases, however, remains unclear. Recently, an estrogen receptor coactivator, AIB1, was identified with higher expression in ovarian cancer cell lines and breast cancer cells than in other cell lines tested (6), implying that increased expression of cofactors might be involved in some hormone-responsive tumors. The question whether cofactors of AR, the major promoter of prostate tumor growth, can also play vital roles for the maintenance of androgen-dependent status is thus of vital interest.

Here we report for the isolation and characterization of a novel AR coactivator, ARA55, which can bind to wild type AR (wtAR) and mutant AR (mAR) in a ligand-dependent manner and enhance their transcriptional activities. The potential roles of ARA55 in prostate cancer is also discussed.

EXPERIMENTAL PROCEDURES

Expression Plasmids—A human prostate cDNA library in pACT2 yeast expression vector was a gift from Dr. S. Elledge. For construction of pP2-wtAR or mAR, C-terminal fragments (aa 595–918) from wtAR or mAR (mART877S, point mutation threonine to serine at codon 877) from Dr. S. P. Balk (7), respectively, were inserted in pAS2 yeast expression vector (CLONTECH). pG5CAT reporter plasmid (CLONTECH) contains five GAL4 binding sites upstream of the E1b TATA box, linked to the CAT gene.

Screening of Prostate cDNA Library by a Yeast Two-hybrid System—A pACT2-prostate cDNA library that consists of the GAL4 activation domain (aa 768–881) fused with human prostate cDNA library was transformed into Y180 yeast cells with a plasmid of pAS2-mAR (mART877S) that contains GALADBD fused with the C-terminal domain of this mAR. Transformants were selected for growth on synthetic dropout (SD) plates with 25 μg 3-aminotriazole and 100 μM dihydrotestosterone (DHT) lacking histidine, leucine, and tryptophan. Colonies were also filter-assayed for β-galactosidase activity. DNAs from positive clones were recovered from yeast, amplified in E. coli, and confirmed by sequencing.

RACE-PCR—The missing 5′-coding region was isolated by 5′-RACE PCR according to the manufacturer’s protocol of Marathon cDNA Amplification Kit (CLONTECH). The gene-specific antisense primer used for 5′-RACE-PCR was 5′-TCAGCCGAAAGCTTACCAGAAGCAGGG-3′. The specific PCR reaction condition was 94 °C for 1 min, 5 cycles of 94 °C for 1 min → 72 °C for 3 min, 5 cycles of 94 °C for 1 min → 70 °C for 3 min, 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.

Co-immunoprecipitation of AR and ARA55—Lysates from in vitro translated full-length AR and ARA55 were incubated with or without
10^{-8} \text{M} \text{DHT} \text{ in the modified RIPA buffer (50 mM Tris-} \text{HCL, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, aprotinin, leupeptin, pepstatin, 0.25% Na-deoxycholate, 0.25% gelatin) and rocked at 4 °C for 2 h. The conjugated beads were washed four times with RIPA buffer, boiled in SDS sample buffer, and analyzed by 8% SDS-polyacrylamide gel electrophoresis and visualized by STORM 840 (Molecular Dynamics).}

Northern Blotting—
The total RNA (25 mg) was fractionated on a 1% formaldehyde-MOPS-agarose gel, transferred onto a Hybond-N nylon membrane (Amersham Pharmacia Biotech) and prehybridized. A probe corresponding to the 900 bp C terminus of ARA55 was 32P-labeled in vitro using Random Primed DNA Labeling Kit (Boehringer Mannheim) according to the manufacturer's protocol and hybridized overnight. After washing, the blot was exposed and quantified by PhosphorImager (Molecular Dynamics). GAPDH was used to monitor the amount of total RNA in each lane.

Transfection Studies—DU145 cells and PC3 cells were grown in Dulbecco’s minimal essential medium (DMEM) containing 5% fetal calf serum (FCS). One hour before transfection, the medium was changed to DMEM containing 5% charcoal-stripped fetal calf serum. Cells were transfected using the modified calcium phosphate technique for 24 h, the medium was changed, and cells were treated with either steroid hormones or hydroxyflutamide (HF) for another 24 h. The cells were harvested, and cell lysates were normalized by β-galactosidase internal control and assayed for chloramphenicol acetyltransferase (CAT) activity. CAT activity was quantified by PhosphorImager.

RT-PCR—For RT-PCR, the total RNA (2 µg) from each sample was reverse-transcribed using SuperScript Preamplification System (Life Technologies, Inc.) in a total reaction volume of 20 µl. cDNA (1 µl) was amplified by PCR with AmpliTaq Gold (Perkin-Elmer). The following sense and antisense primers were used: sense primer, 5'-GCACTTCGTTTGCGGAGGC-3'; antisense primer, 5'-CCGAAGAGCTTCAGGAAAGC-3'. This combination of primers amplifies 633 bp of the C-terminal region of ARA55. After an initial denaturation at 95 °C for 9 min, 33 cycles of amplification (denaturation at 94 °C for 9 min, annealing at 63 °C for 1 min, and extension at 72 °C for 1 min) were followed by a terminal extension at 72 °C for 1 min. PCR products were visualized on a 1% agarose gel containing ethidium bromide. The 600-bp fragment of GAPDH, which was amplified with 30 amplification cycles using the sense and antisense primers for human GAPDH (Stratagene), was used to demonstrate comparable RNA amounts and quality among samples.

RESULTS
Cloning and Sequencing of ARA55—Loss of androgen specificity in mAR may contribute to the development of prostate cancer from an androgen-dependent to androgen-independent state (7, 8). We were interested to know if wtAR and mAR...
might exert their functions by recruiting additional cofactors. A yeast two-hybrid system with mART877S as a bait was used to screen the human prostate cDNA library. As a result, six clones that interacted with mART877S were isolated, and one of them, named ARA55, was further characterized because its DNA sequence was highly homologous to the C terminus of mouse hic5 (a hydrogen peroxide-inducible clone) (9).

Northern blot analysis indicated that ARA55 mRNA, with a size near 2 kilobases, could be detected in HeLa and prostate PC-3 cells but not in other cell lines such as HepG2, H1299, MCF7, CHO, PC12, P19, and DU145 (data not shown). The 5'-RACE-PCR technique was then used to clone the full-length ARA55 from HeLa cells. Sequence analysis determined that the open reading frame between the first ATG and terminal TGA encoded 444 aa for human ARA55 with the predicted molecular mass of 55 kDa (Fig. 1A). Amino acid sequence analysis indicated that human ARA55 shares 90.5% homology with mouse Hic5 (9). Another interesting finding from this deduced aa sequence was the existence of four LIM motifs in the C-terminal regions (Fig. 1B). The LIM motif is a cysteine-rich zinc-binding motif with the consensus sequence: $\text{CX}_2\text{CX}_{16-23}\text{HX}_2\text{CX}_{2}\text{CX}_{16-21}\text{CX}_{2}(\text{C, H, D})$ (10). Although the function of the LIM motif has not been fully defined, some data suggest that it might be involved in the protein-protein interaction (11). Among all identified SR-associated proteins, only the thyroid hormone interacting protein 6 (Trip6) has these similar LIM motifs (12).

Interaction between ARA55 and AR Is Ligand-dependent—We first tested whether the interaction between ARA55 and AR is ligand-dependent in the yeast and mammalian systems. GAL4 AR, which contains the GAL4 DBD fused to the C terminus (aa 595–918) of wtAR or mART877S (pAS2-wtAR or -mAR), was transformed into Y190 yeast cells with a plasmid of GAL4 activation domain fused to ARA55251–444 (pACT2-ARA55). Transformants were selected for growth on the SD plates with serial concentrations of steroid hormones lacking histidine, leucine, and tryptophan. Colonies were also filter-assayed for β-galactosidase activity. T, testosterone; P, progesterone; Dex, dexamethasone; B, the ability of a fusion protein, comprising the full-length ARA55 and the activation domain of VP16 (VP16-ARA55), to interact with the hormone binding domain of AR fused to the GAL4 DBD (GAL0AR) was examined in DU145 cells by determining the CAT activity from the reporter plasmid pSG5CAT. Ten nM DHT was used as a ligand. C, immunoprecipitation of AR and ARA55. The in vitro translated pET-ARA55 and AR incubated with or without 10 nM DHT were shown from lanes 1–4. The polyclonal anti-His/Tag antibodies were used for co-immunoprecipitation, and 10 μl of protein A/G-Sepharose beads were applied to precipitate the protein-antibody complex. The in vitro translated AR was used in lane 1 as an input control. Molecular mass markers are in kDa.

**Fig. 3.** Full-length ARA55 enhances AR transcriptional activity. DU145 cells were transiently co-transfected with 3 μg of MMTV-CAT reporter plasmid, with or without 1 μg of AR expression vector (pSG5AR), increasing amounts of full-length ARA55 expression vectors (pSG5ARA55) as indicated, or 5 μg of C-terminal (aa 251–444) region of ARA55 vectors (pSG5ARA55251–444), either in the presence or absence of 10 nM DHT. Each relative CAT activity is presented relative to the transactivation observed in the absence of ARA55 and in the presence of 10 nM DHT (lane 1). Bars represent the mean ± S.D. of three independent experiments.
not promote this interaction. There were no differences between wtAR and mAR in their interactions with ARA55 (data not shown). Together, these data suggested that ARA55 can interact with not only mAR, but also wtAR in the presence of DHT, testosterone, and higher concentrations of E2 or progesterone and that the presence of only the C-terminal region of ARA55 is the minimal requirement for interaction.

Next, we tested this interaction in a mammalian two-hybrid system. DU145 cells were co-transfected with a plasmid encoding the hormone binding domain of wtAR fused to the GAL4 DBD (GAL0AR) and with a plasmid encoding full-length ARA55 fused to the activation domain of VP16 (VP16-ARA55). Interaction was estimated by determining the level of CAT activity from the reporter plasmid (Fig. 2B). A combination of GAL0 empty vector and VP16-ARA55 did not show any CAT activity (Fig. 2B, lane 1). A combination of GAL0AR and VP16 vector showed a negligible amount of CAT activity (Fig. 2B, lane 2), and a significant level of CAT activity was induced by the co-transfection of VP16-ARA55 and GAL0AR only in the presence of 10 nM DHT (Fig. 2B, lane 3 versus lane 4). These results indicate that ARA55 can interact with AR and that this interaction is ligand-dependent.

To further prove that the interaction between AR and ARA55 is the direct interaction, a co-immunoprecipitation assay was performed using an in vitro transcription/translation system, which expressed high levels of AR and His-Tag fusion ARA55. A polyclonal anti-His-Tag antibody was employed for co-immunoprecipitations, and the resulting immune complexes were subjected to SDS-polyacrylamide gel electrophoresis. As shown in Fig. 2C, the AR can only be co-immunoprecipitated by incubation with ARA55 that is fused with His-Tag, and the addition of $10^{-8}$ M DHT enhances significantly the specific interaction between AR and ARA55 (Fig. 2C, lane 3 versus lane 4).

ARA55 Enhances AR Transcriptional Activity—Because yeast and mammalian two-hybrid systems and co-immunoprecipitations all indicated that ARA55 can interact with AR in a DHT-dependent manner, we were then interested in knowing if such an interaction can affect further AR transcriptional activity. As shown in Fig. 3, the ligand-free AR had only minimal MMTV-CAT reporter activity with or without the ARA55, and ARA55 alone also showed only minimal reporter activity (Fig. 3, lane 4). However, addition of 10 nM DHT resulted in a 4.3-fold increase of AR transcriptional activity and ARA55 further increased this induction by 5.3-fold (from 4.3-fold up to 22.8-fold) in a dose-dependent manner (Fig. 3, lanes 5–9). The induced activity reached a plateau at the ratio of AR:ARA55 to 1:4.5. A similar induction result could also be obtained when we replaced DU145 cells with PC-3 cells, or MMTV-CAT with a PSA-CAT reporter plasmid that had a 2.8-kilobase promoter region of PSA gene containing androgen responsive regions (data not shown).

The Effects of ARA55 on the Transcriptional Activities of wtAR and mARs—We then examined the effect of ARA55 on two different mARs in the presence of serial concentrations of DHT, testosterone, and higher concentrations of E2 or progesterone. DU145 cells were transiently co-transfected with 1 μg of wtAR (pCMVAR), LNCaP mutant AR (pCMVm-ART877A), or mARE708K (pCMVmARE-708K), and 4.5 μg of ARA55, or empty expression vector in the presence or absence of DHT, E2, or HF, at the indicated concentrations. Three μg of MMTV-CAT plasmid was used as a reporter. Each relative CAT activity is presented relative to the transactivation observed in the absence of ligand. Bars represent the mean ± S.D. of three independent experiments.
ARA55 is an AR Coactivator

The activities of AR, GR, PR, and ER. DU145 cells were transiently cotransfected with 3 μg of reporter plasmids (MMTV-CAT for AR, GR, and PR, and ERE-CAT for ER), 1 μg of each receptor in pSG5, and 4.5 μg of ARA55 (or 3 μg of ARA70), or empty pSG5 vector, in the presence of 10 nM cognate ligand. Relative CAT activity is presented relative to the transactivation observed in the absence of ARA55. Bars represent the mean ± S.D. of at least four independent experiments.

Fig. 5. The effects of ARA55 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, and ERE-CAT for ER), 1 μg of each receptor in pSG5, and 4.5 μg of ARA55 (or 3 μg of ARA70), or empty pSG5 vector, in the presence of 10 nM cognate ligand. Relative CAT activity is presented relative to the transactivation observed in the absence of ARA55. Bars represent the mean ± S.D. of at least four independent experiments.

Fig. 6. ARA55 mRNA expression in the prostate cancer cell lines and clinical prostate samples. Northern blotting and RT-PCR were performed using total RNA from each sample as described under “Experimental Procedures.” A, Northern blotting of prostate cancer cell lines. Lane 1, PC-3; lane 2, DU145; lane 3, LNCaP; GAPDH was used as a control. B, RT-PCR of prostate cancer cell lines and human prostate samples. The expected RT-PCR product of ARA55 was 633 bp; M, 1-kb ladder (Life Technologies, Inc.); lane 1, PC-3; lane 2, DU145; lane 3, LNCaP; lane 4, normal prostate; lane 5, benign prostatic hypertrophy; lanes 6–11, prostate cancer. GAPDH was used as a control.

ARA55 Expression in the Prostate—The mARs (mART777S and mART877A) were identified in advanced prostate cancer patients and could respond to antiandrogens or estrogen as well as androgen. Because ARA55 could enhance the transactivation of these mARs as well as wtAR, we were interested in knowing whether the prostate cancer cells or the prostate tissue could express the ARA55. Northern blot analysis demonstrated that DU145 and LNCaP cells did not, but PC-3 cells did express ARA55 mRNA (Fig. 6A). Next, we examined ARA55 mRNA expression in the clinical prostate samples and the cell lines, using RT-PCR (Fig. 6B), because these clinical samples were obtained by needle biopsy and the amount of the extracted RNA was not sufficient for Northern blotting analysis. As the Northern blotting demonstrated, PC-3 cells expressed ARA55 mRNA, but DU145 and LNCaP cells did not. Every clinical prostate sample, including normal, benign hypertrophy, and cancer, expressed ARA55 mRNA, but there were some differences in its expression level among the samples. This heterogeneity of ARA55 expression might be one of the factors that cause the different clinical courses of prostate cancer.

Discussion

The unique sequence of ARA55 places this AR coactivator outside the family of common SR coactivators that include SRC-1 (17), TIF2/GRIP1 (18, 19), and ACTR/AIB1/RAC3 (4, 6, 20). For example, ARA55 lacks some common motifs (such as basic helix-loop-helix (bHLH) domain and Per-AhR-Sim (PAS) domain) that are shared by SR coactivators. Instead, ARA55 does have three LIM motifs in the interaction domain of the C-terminal region. The LIM motif is a cysteine-rich motif that is found in several proteins (including Trip 6), with diverse functions and subcellular distributions. The biochemical properties and the function of the LIM motifs have not been fully defined, and it has been suggested that their main function is in developmental regulation (10, 21). However, Schmeichel and Beckerle reported that LIM motifs might be involved in protein-protein interaction (11). Therefore, the LIM motifs in the C-terminal region of ARA55 may contribute to its interaction with AR.

There is also no homology between ARA55 and the first identified AR coactivator, ARA70 (16, 22–26). Although both...
AR coactivators enhance AR transcriptional activity in DU145 cells, they show distinct differences: 1) ARA55 is more general to SRs whereas ARA70 is more specific to AR; 2) ARA55 has a lesser effect than ARA70 on AR-mediated transactivation in the presence of E2 and HF; and 3) ARA55 is a TGF-β1-inducible gene. The precise role of these two cofactors may affect the different physiological influences on prostate cells.

One of the very interesting features of ARA55 sequence is its high homology to mouse hic5, a well documented TGF-β1-inducible gene (9). Whereas previous studies suggested that hic5 may play important roles in the growth-inhibitory pathway associated with senescence (27), the linkage of hic5 to steroid hormones and their receptors has not been demonstrated. Our findings here may therefore provide a potential connection to allow TGF-β1 to increase AR transcriptional activity via induction of ARA55 in prostate.

Prostate cancer is the most frequently diagnosed malignant tumor and the second leading cause of cancer death in American men (28). Today, the only effective treatment for advanced prostate cancer is hormonal therapy that combines surgical or chemical castration with administration of antiandrogens, such as HF or E2. Although hormonal therapy is very effective and most of the patients initially respond to this treatment, the vast majority of them may relapse within 18 months (29). The mechanisms by which prostate cancer cells become resistant to hormonal therapy remain unclear. One popular hypothesis to explain how prostate can progress from androgen independent-state is that mutations in AR may change this receptor's sensitivity to other steroid hormones or antiandrogens, such as E2 and HF. As ARA55 was able to induce transcriptional activity of both wtAR and mAR in the presence of DHT, E2, and HF, and ARA55 was expressed differently in various prostate tumor cells, ARA55 may therefore, play some important roles for the progression of prostate cancer and its resistance to hormonal therapy. Further studies of blocking the function of ARA55 (e.g. a small peptide or compound that can interfere with the interaction of AR and ARA55) may provide a novel clue for the development of treatments of the advanced prostate cancer.

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