

A Dominant-negative Mutant of Androgen Receptor Coregulator ARA54 Inhibits Androgen Receptor-mediated Prostate Cancer Growth*

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The ligand-bound androgen receptor (AR) regulates target genes via a mechanism involving coregulators such as androgen receptor-associated 54 (ARA54). We investigated whether the interruption of the AR coregulator function could lead to down-regulation of AR activity. Using *in vitro* mutagenesis and a yeast two-hybrid screening assay, we have isolated a mutant ARA54 (mt-ARA54) carrying a point mutation at amino acid 472 changing a glutamic acid to lysine, which acts as a dominant-negative inhibitor of AR transactivation. In transient transfection assays of prostate cancer cell lines, the mt-ARA54 suppressed endogenous mutated AR-mediated and exogenous wild-type AR-mediated transactivation in LNCaP and PC-3 cells, respectively. In DU145 cells, the mt-ARA54 suppressed exogenous ARA54 but not other coregulators, such as ARA55-enhanced or SRC-1-enhanced AR transactivation. In the LNCaP cells stably transfected with the plasmids encoding the mt-ARA54 under the doxycycline inducible system, the overexpression of the mt-ARA54 inhibited cell growth and endogenous expression of prostate-specific antigen. Mammalian two-hybrid assays further demonstrated that the mt-ARA54 can disrupt the interaction between wild-type ARA54 molecules, suggesting that ARA54 dimerization or oligomerization may play an essential role in the enhancement of AR transactivation. Together, our results demonstrate that a dominant-negative AR coregulator can suppress AR transactivation and cell proliferation in prostate cancer cells. Further studies may provide a new therapeutic approach for blocking AR-mediated prostate cancer growth.

There is a substantial amount of evidence to indicate that steroid hormone receptors function as a tripartite system involving the receptor, its ligands, and its coregulator proteins (1–5). The androgen receptor (AR),¹ a member of this receptor superfamily, is a ligand-dependent transcriptional factor that

mediates the biological effects of androgens in a variety of target tissues including the prostate. AR involvement is also associated with a number of pathological conditions, notably prostate cancer (6–9). Recently, a number of steroid receptor coactivators including steroid receptor coactivator-1 (SRC-1) (10), GRIP1/TIF2 (11, 12), pCIP/ACTR/AIB1/RAC3/TRAM-1 (13–16), TIF1 (17), RIP140 (18), TAFII30 (19), PGC-1 (20), SNURF (21), and others (2, 3, 22) has been identified as being able to modulate steroid receptor transactivation. We have also isolated and characterized several coregulators as AR-associated (ARA) proteins that enhance AR transcriptional activation by interacting with AR in a ligand-dependent manner (23–29). One of the AR coregulators, ARA54, can enhance the transactivation of wild-type AR and a mutant AR derived from LNCaP prostate cancer cells in prostate cancer cells by 2–6-fold in the presence of androgens or the antiandrogen hydroxyflutamide (HF) (26, 29).

Prostate cancer is the most frequently diagnosed malignancy in aging males. In 2001, it is estimated that there will be 198,100 new cases of prostate cancer and 31,500 deaths from this disease in the United States. (30). The most significant palliative treatment of prostate cancer is hormonal therapy involving androgen ablation by surgical or medical castration and/or the administration of antiandrogens. Nonetheless, no curative treatment exists for patients with metastatic prostate cancer. Indeed, most of the patients with advanced prostate cancer eventually develop androgen-independent disease. Additionally, some patients with androgen-dependent disease develop a withdrawal syndrome that is associated with an agonist effect of antiandrogens, resulting in antiandrogen treatment promoting prostate cancer progression (31). Our previous studies have suggested that AR coactivators promote the agonist activity of antiandrogens through the interaction with AR (5, 29, 32). The interruption of this AR-coregulator interaction may therefore provide a target for the development of novel treatment strategies for advanced prostate cancer.

In our previous study (26), the C-terminal region (amino acids 361–474) of ARA54 (C'-ARA54), which was originally isolated from a human prostate cDNA library, interacted with AR. We found that full-length ARA54 (fl-ARA54) but not C'-ARA54 enhanced AR transactivation (26, 29). This study was undertaken to search for a potential strategy that can suppress AR transactivation induced by fl-ARA54 in prostate cancer cells. We hypothesized that mutant ARA54, which has lost the ability to bind to AR, might be able to act as a dominant-

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¹ The abbreviations used are: AR, androgen receptor; ARA, androgen receptor-associated; HF, hydroxyflutamide; C'-ARA54, C-terminal region of ARA54; fl-ARA54, full-length ARA54; mt-ARA54, mutant ARA54; DHT, 5 α -dihydrotestosterone; CAT, chloramphenicol acetyltransferase; Luc, luciferase; PSA, prostate-specific antigen; GR, glu-

cocorticoid receptor; PR, progesterone receptor; doxy, doxycycline; MMTV, mouse mammary tumor virus; RB, retinoblastoma; SRC-1, steroid receptor coactivator-1.

negative inhibitor of AR transcription. Using a chemical mutagenesis method to create a mutated C'-ARA54 library for two-hybrid screening in yeast, we isolated a mutant ARA54 (mt-ARA54), a C-terminal fragment of ARA54 with a point mutation that functions in a dominant-negative manner. This dominant-negative clone disrupts the ability of wild-type ARA54 to interact with itself, suggesting that ARA54 dimerization or oligomerization may play an important role in the enhancement of AR transactivation. The hydroxylamine-mediated mutagenesis screening technique used in this study can be used to isolate additional dominant-negative coregulators that are able to inhibit a broad spectrum of receptor-coregulator interactions. Such dominant-negative coregulators may ultimately be used in gene therapy as part of a therapeutic option in the treatment of prostate cancer.

EXPERIMENTAL PROCEDURES

Chemicals and Plasmids—5 α -Dihydrotestosterone (DHT), progesterone, and dexamethasone were obtained from Sigma, and HF was from Schering. pAS2-AR containing the C terminus of the ligand binding domain from wild-type AR fused to the GAL4 DNA binding domain (DBD) was constructed as described previously (25). pACT2-C'-ARA54 fused with the GAL4 activation domain was the clone identified originally from prostate cDNA library (26). pSG5-AR, pSG5-C'-ARA54, pSG5-fl-ARA54, pSG5-ARA55, pSG5-ARA70, and pSG5-SRC-1 were constructed as described previously (4, 23, 25, 26). pSV-mutant AR877 (33) and pSG5-Rb were provided by Drs. S. Balk and W. Kaelin, Jr., respectively. pGAL0-AR containing the AR ligand binding domain fused with the GAL4 DBD and pCMX-VP16-fl-ARA54 fused to the activation domain of VP16 were constructed as described previously (26, 29). pCMX-GAL4 DBD-fl-ARA54 was constructed by inserting the *EcoRI/SacI* fragment of ARA54 in frame to the GAL4DBD. pCMX-VP16-C'-ARA54 and pCMX-VP16-mt-ARA54 were constructed using the C'-ARA54 and mt-ARA54 *BamHI* fragments.

Mutated Library Construction—An ARA54-mutated library was generated by incubating 100 μ g of pACT2-C'-ARA54 with 1 M hydroxylamine (Sigma) at 70 $^{\circ}$ C for 1 h followed by DNA extraction.

Yeast Two-Hybrid Screening—Plasmids with pAS2-AR and the mutated ARA54 library were sequentially transformed into the yeast strain Y190 harboring reporter genes (*i.e.* *lacZ* and *His-3*) according to the CLONTECH Yeast Protocols Handbook. The transformed yeast cells were plated with 100 nM DHT on synthetic dropout plates lacking tryptophan and leucine. Colonies were filter-assayed for β -galactosidase activity, and white colonies that indicated no interaction between the AR bait and mutant ARA54 were selected. The mutant ARA54 plasmid DNAs were isolated from the yeast cells that have spontaneously lost the cycloheximide-bearing plasmid (pAS2-AR) by plating the selected white colonies on synthetic dropout (-leucine) in the presence of 10 μ g/ml cycloheximide (Sigma). The mutant ARA54 clones were then subcloned into the pSG5 mammalian expression vector (Stratagene).

Cell Culture, Transient Transfections, and Reporter Gene Assays—The human prostate cancer cell lines, LNCaP, PC-3, and DU145, were maintained in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. Transfections using the calcium phosphate precipitation method and chloramphenicol acetyltransferase (CAT) and luciferase (Luc) assays were performed as described previously (5, 29, 34). 1–4 \times 10⁵ cells were plated on 35- or 60-mm dishes 24 h before adding the precipitation mix containing a CAT or Luc reporter gene and a β -galactosidase expression plasmid (pCMV- β -galactosidase) as an internal control for normalization of transfection efficiency. The medium was changed to phenol-red-free Dulbecco's modified Eagle's medium with 5% charcoal-stripped fetal calf serum 1 h before transfection. In each experiment, the total amount of transfected DNA/dish was maintained as a constant by the addition of empty expression vector (pSG5 or pVP16, as appropriate). The medium was changed again 24 h after transfection, and the cells were treated with 1 nM DHT or 1 μ M HF for 24 h. The cells were then harvested, and whole cell extracts were used for CAT or Luc assay. The CAT activity was quantitated with a PhosphorImager (Molecular Dynamics). The Luc assay was determined using a Dual-Luciferase Reporter Assay System (Promega) and luminometer.

Establishment of LNCaP Cell Lines Stably Transfected with the Plasmids Encoding the Mutant ARA54 under the Inducible Promoter—The pBIG2i vector contains all of the elements required for tetracycline-responsive gene expression and a selective marker conferring resist-

ance to hygromycin B for the generation of stable cell lines (35). We first constructed pBIG2i-C'-ARA54, pBIG2i-mt-ARA54, and pBIG2i-fl-ARA54 and then transfected each plasmid into LNCaP or PC-3 cells using SuperFect transfection reagent (Qiagen). After transfection, cells were cultured in the presence of 100 μ g/ml hygromycin B (Invitrogen) to select for stably transfected cells that had incorporated the pBIG2i-based construct. After growth for an additional two weeks, individual clones were picked. We then confirmed the stable expression of the mutant (C-terminal fragment) or wild-type (full-length) ARA54 induced by doxycycline using Northern blot analysis. Northern blotting was performed using total RNAs from the stable LNCaP or PC-3 cells and C-terminal fragment of ARA54 as a DNA probe as described previously (25, 26).

Western Blot—Western blotting analysis was performed in the stable LNCaP cells using NH27 polyclonal antibody for the AR and monoclonal prostate-specific antigen (PSA) antibody (DAKO) as described previously (5). An antibody for β -actin (Santa Cruz Biotechnology) was used as the internal control.

Mammalian Two-hybrid Assay—DU145 cells were transiently co-transfected with a GAL4-hybrid expression plasmid, a VP16-hybrid expression plasmid, the reporter plasmid pG5-CAT, and the pCMV- β -galactosidase internal control plasmid. Transfections and CAT assays were performed as described above.

RESULTS

Isolation of Dominant-Negative Mutant ARA54—To screen for dominant-negative forms of ARA54, we used an *in vitro* mutagenesis strategy combined with the yeast two-hybrid system. ARA54 was initially isolated from a human prostate cDNA library as a C-terminal fragment that interacted with AR (26). This C-terminal region of ARA54 (amino acids 361–474) was cloned into pACT2 and mutagenized with 1 M hydroxylamine to create the mutant ARA54 library for yeast two-hybrid screening. This library was screened against pAS2-AR for the selection of clones that did not interact with AR. We selected 11 colonies that showed no interaction between pAS2-AR and the pACT2-ARA54 mutant from ~50,000 yeast colonies. We confirmed the interactions with AR by subcloning each clone into pACT2 and yeast two-hybrid assay with sequential transformation with pAS2-AR and pACT2 mutant clone. These 11 pACT2 constructs were then subcloned into pSG5 to assess their effect on AR-mediated transactivation in the prostate cancer cell lines LNCaP (AR-positive and ARA54-positive), PC-3 (AR-negative and ARA54-positive), and DU145 (AR-negative and ARA54-negative) (26) using a reporter gene assay. We have previously shown that the transcriptional activity of a mutant AR or wild-type AR could be induced in LNCaP or PC-3 cells in response to both androgen, DHT, and the antiandrogen, HF, and that fl-ARA54 can enhance the AR transactivation in DU145 cells (26, 29, 34, 36, 37). Fig. 1 shows that C'-ARA54 suppresses DHT-mediated or HF-mediated AR transcriptional activity. One mutant ARA54 clone was found to have a stronger dominant-negative effect both for endogenous fl-ARA54 in LNCaP and PC-3 cells and for exogenous fl-ARA54 in DU145 cells. However, both mutants, C'-ARA54 and mt-ARA54, showed an only marginal effect on AR transactivation in the absence of fl-ARA54 in DU145 cells (Fig. 1, E and F). The suppression of AR transactivation by either C'-ARA54 or mt-ARA54 was not the result of down-regulation of AR protein expression. LNCaP cells transfected with C'-ARA54 or mt-ARA54 showed little change in endogenous AR expression compared with non-transfected cells (data not shown). These results suggest that a mutant ARA54 dominant-negatively suppresses endogenous AR-mediated and exogenous AR-mediated transactivation. Sequencing analysis revealed that mt-ARA54 contained a single point mutation (a guanine to adenine transition) at the first position of codon 472, resulting in a glutamic acid to lysine substitution.

Effect of the Dominant-Negative ARA54 Mutant on the Transactivation Mediated by Different Steroid Receptors—

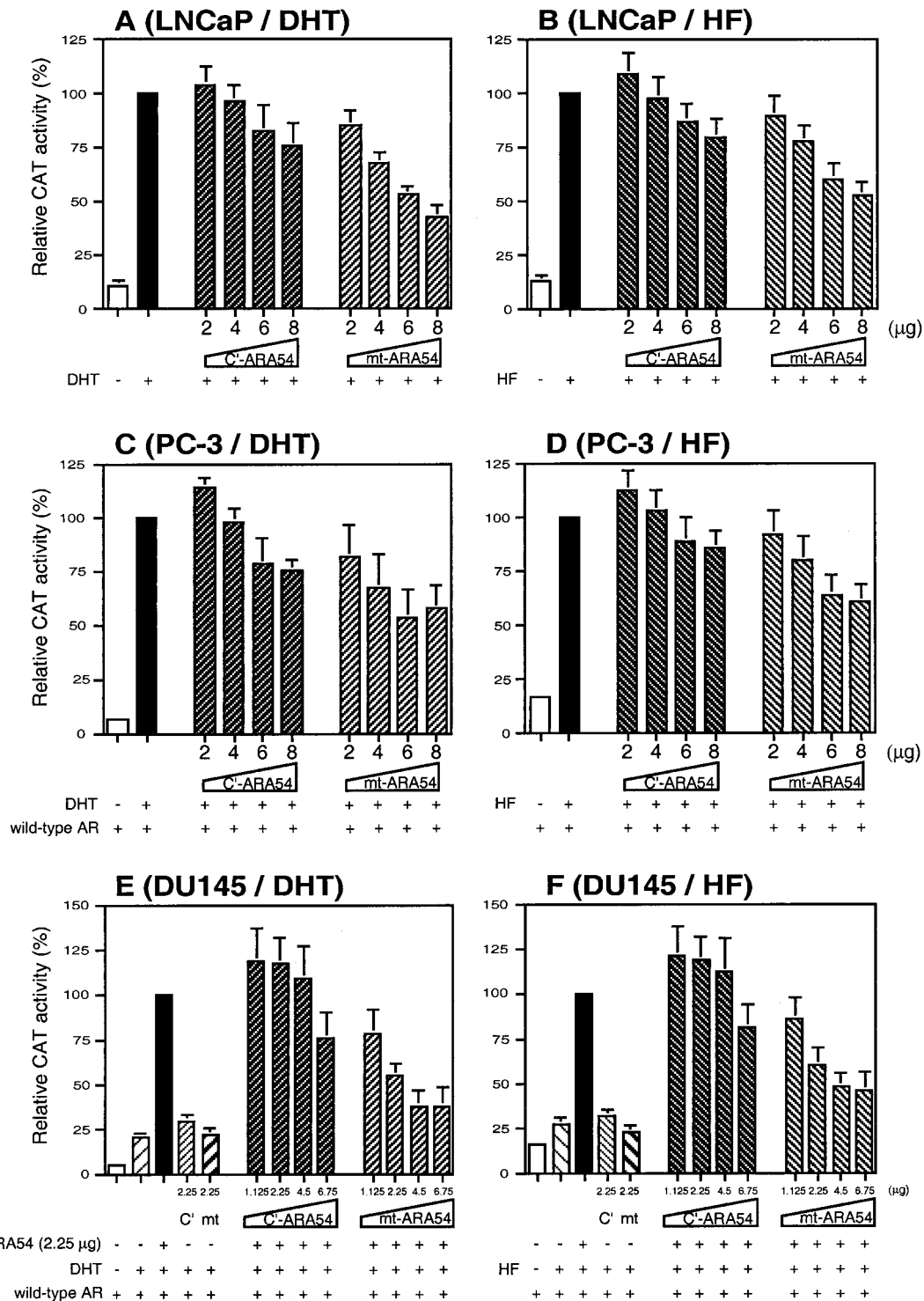


FIG. 1. The dominant-negative effects of C'-ARA54 and mt-ARA54 on AR transcriptional activity in human prostate cancer cell lines. LNCaP (A and B), PC-3 (C and D), or DU145 (E and F) cells were transfected with mouse mammary tumor virus (MMTV)-CAT plasmid (2.5 μg) and increasing amounts of pSG5-C'-ARA54 or pSG5-mt-ARA54 as indicated. The wild-type AR expression plasmid pSG5-AR was cotransfected in PC-3 and DU145 cells (1.0 μg for PC-3 and 0.75 μg for DU145). DU145 cells were also transfected with 2.25 μg of pSG5-fl-ARA54. The total amount of DNA was adjusted to 11.5–13.25 μg with pSG5 for each transfection. 24 h after transfection, cells were cultured for an additional 24 h in the presence or absence of 1 nM DHT (A, C, and E) or 1 μM HF (B, D, and F). The CAT activity is presented relative to that of vector alone with DHT or HF in each panel (black bars, set as 100%). Values represent the mean ± S.D. of at least three determinations.

Our previous studies demonstrated ARA54 had a marginal transcriptional effect on the glucocorticoid receptor (GR) but could enhance the transcriptional activity of the progesterone receptor (PR) by up to 4-fold (26). We examined the effect of mt-ARA54 on PR and GR transactivation in the presence of endogenous or exogenous fl-ARA54. Both C'-ARA54 and mt-ARA54 had only a marginal effect on PR-mediated transac-

tivation in the presence of progesterone in the PC-3 cell line. Similarly, GR transactivation was only marginally repressed by either C'-ARA54 or mt-ARA54 (Fig. 2A). When fl-ARA54 was cotransfected with PR or GR into DU145 cells, fl-ARA54 induced PR transcription by 2.9-fold and GR transcriptional activity by 1.6-fold (Fig. 2B). In DU145 cells, mt-ARA54 suppressed fl-ARA54-induced PR transactivation by 43% but

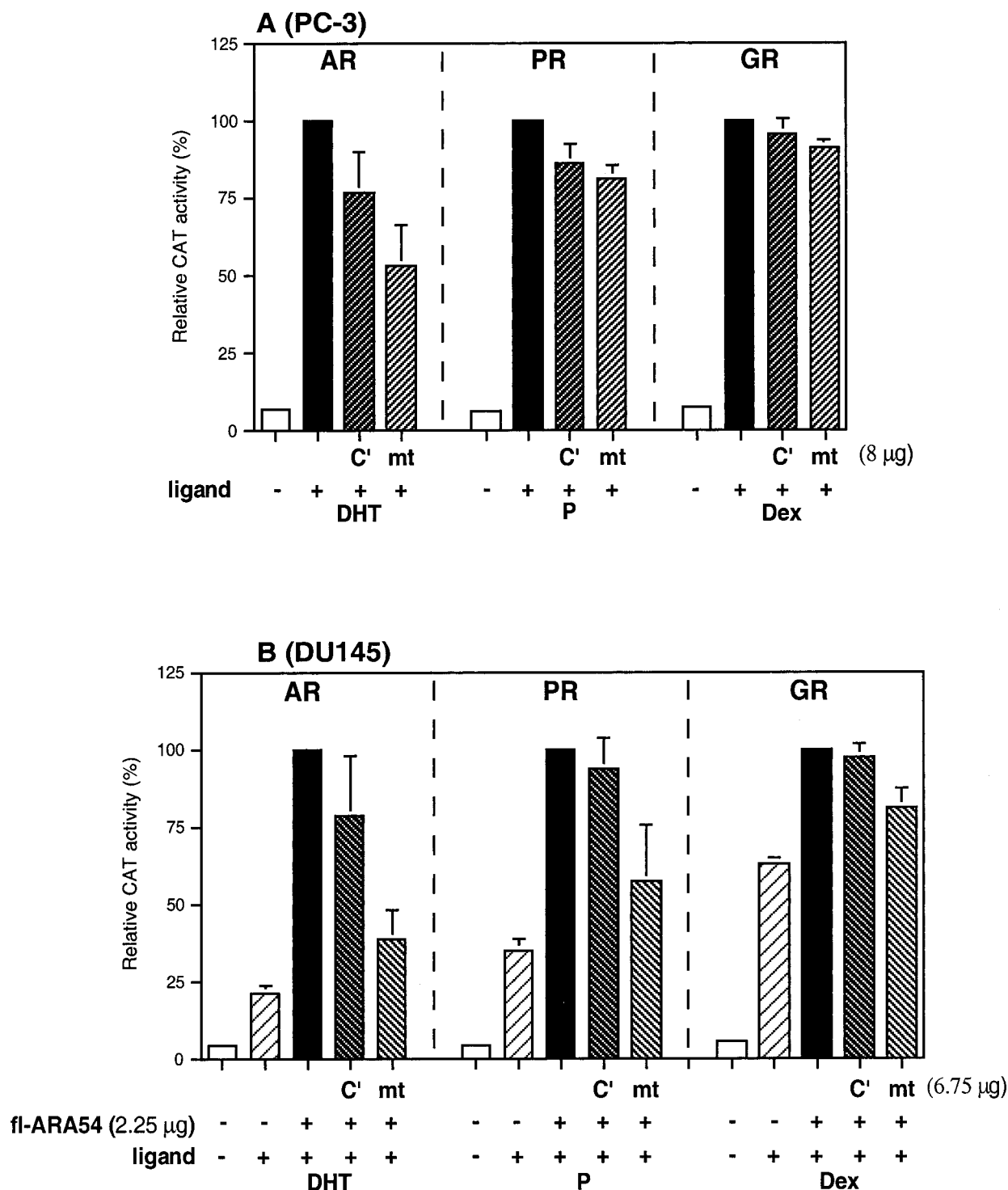


FIG. 2. The dominant-negative effects of C'-ARA54 and mt-ARA54 on the transcriptional activity of AR, PR, and GR. PC-3 (A) or DU145 (B) cells were transfected with MMTV-CAT (2.5 µg), steroid receptor expression plasmid (AR, PR, or GR) (1.0 µg for PC-3 and 0.75 µg for DU145), and pSG5-C'-ARA54 (C') or pSG5-mt-ARA54 (mt) (8.0 µg for PC-3 and 6.75 µg for DU145) with pSG5-fl-ARA54 (2.25 µg) (for DU145) or without pSG5-fl-ARA54 (2.25 µg) (for PC-3). The total amount of DNA was adjusted to 12.5–13.25 µg with pSG5 for each transfection. 24 h after transfection, cells were cultured for an additional 24 h in the presence or absence of 1 nM DHT, 10 nM progesterone, or 10 nM dexamethasone (Dex) as indicated. The CAT activity is presented relative to that of vector alone with cognate ligand in each panel (black bars, set as 100%). Values represent the mean ± S.D. of at least three determinations.

only marginally suppressed GR transactivation. C'-ARA54 showed little effect on PR or GR transcription.

Coregulator Specificity of the Dominant-Negative ARA54 Mutant—To determine whether C'-ARA54 and mt-ARA54 inhibited only wild-type ARA54-mediated transactivation, we examined their effect in DU145 cells in the presence of other AR

coregulators. C'-ARA54 or mt-ARA54 was cotransfected with AR and ARA55, ARA70, retinoblastoma (Rb), or SRC-1 into DU145 cells. As shown in Fig. 3A and consistent with our previous reports (23–26, 29), these coactivators alone enhanced AR transcriptional activity by an additional 2.9–6.0-fold in the presence of DHT. C'-ARA54 and mt-ARA54 showed only mar-

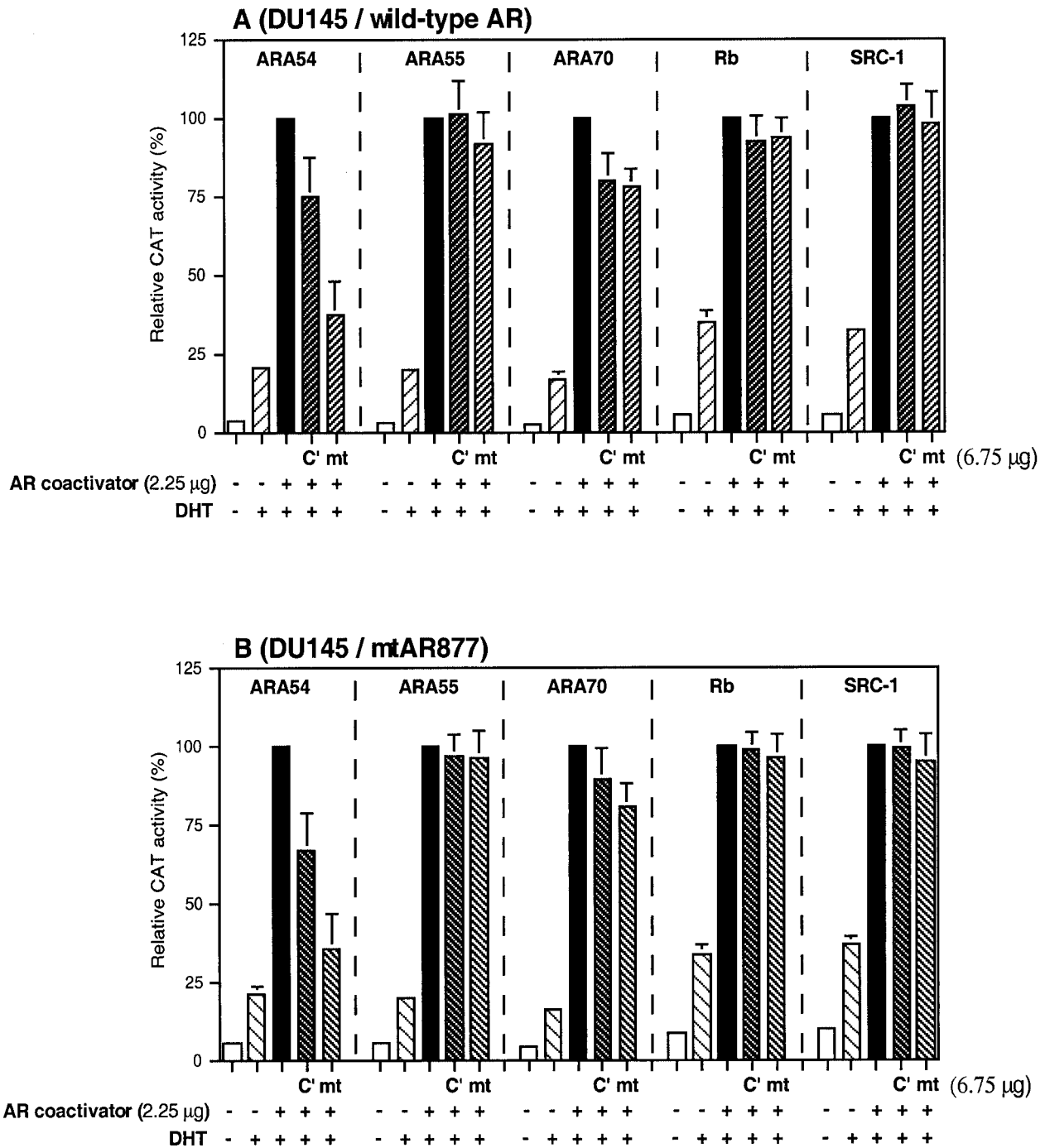


FIG. 3. The effects of C'-ARA54 and mt-ARA54 on AR transcriptional activity in the presence of different AR coactivators. DU145 cells were transfected with 2.5 μg of MMTV-CAT, 0.75 μg of AR expression plasmid (wild-type (A) and mtAR877 (B)), 2.25 μg of different AR coactivators (ARA54, ARA55, ARA70, Rb, or SRC-1), and 6.75 μg of pSG5-C'-ARA54 (C') or pSG5-mt-ARA54 (mt). The total amount of DNA was adjusted to 13.25 μg with pSG5 for each transfection. Twenty-four h after transfection, cells were cultured for an additional 24 h in the presence or absence of 1 nM DHT as indicated. The CAT activity is presented relative to that of vector alone with DHT in each panel (black bars, set as 100%). Values represent the mean ± S.D. of at least three determinations.

ginal or slight suppressive effects on ARA55-, ARA70-, Rb-, or SRC-1-enhanced AR transactivation. Similar results were also obtained when mt-AR877, codon 877 mutation threonine to serine derived from a prostate cancer (33), was substituted for wild-type AR (Fig. 3B). These results suggest that the suppressive effect of mt-ARA54 or C'-ARA54 is relatively specific for fl-ARA54-enhanced AR transactivation.

Effect of the Dominant-Negative ARA54 Mutant on Growth of Prostate Cancer Cells and PSA Expression—To develop a system that allows us to investigate the effect of the dominant-

negative ARA54 mutant on cell proliferation, we have established prostate cancer cell lines stably transfected with the plasmids encoding the mutant ARA54 (C'-ARA54 or mt-ARA54) or fl-ARA54 under the doxycycline (doxy)-inducible promoter. We first confirmed the stable expression of the ARA54 induced by doxy using Northern blotting (data not shown). The LNCaP or PC-3 cell-expressed endogenous ARA54 (wild-type) bands appeared at 3 kb, and strong shorter bands (2 kb) suggestive of C-terminal fragment transcript (C'-ARA54 or mt-ARA54) were detected only in the presence of doxy. Simi-

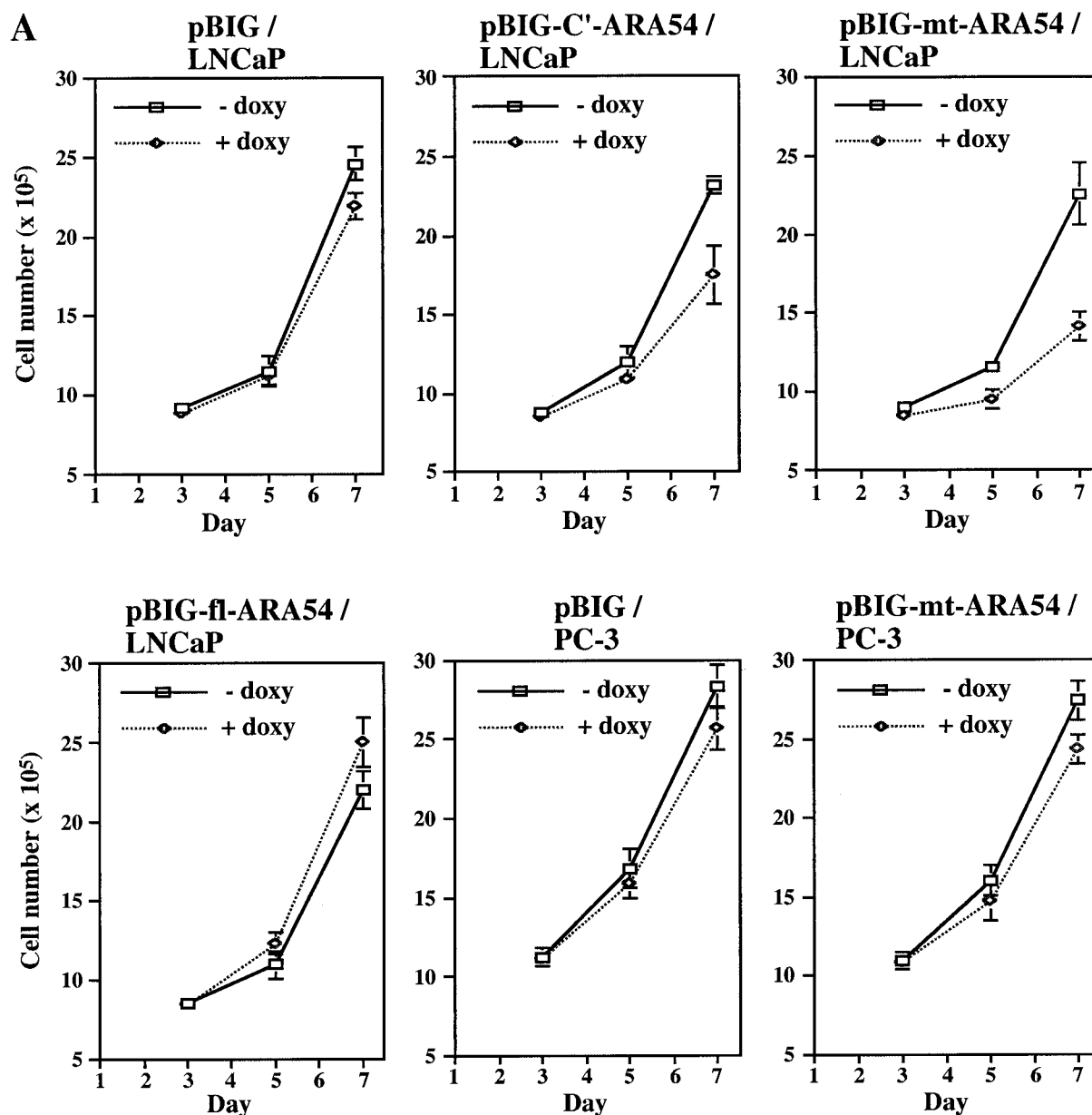


FIG. 4. The effects of the mutant ARA54 in the LNCaP cells stably transfected with pBIG2i-C'-ARA54 or pBIG2i-mt-ARA54 under tetracycline-inducible system. A, the effects of C'-ARA54 and mt-ARA54 on cell proliferation. LNCaP cells stably transfected with pBIG2i (vector alone), pBIG2i-C'-ARA54, pBIG2i-mt-ARA54, or pBIG2i-fl-ARA54, and PC-3 cells stably transfected with pBIG2i (vector alone) or pBIG2i-fl-ARA54 were cultured in the presence or absence of 2 μ g/ml doxy with 1 nM DHT. Total cell number was counted by hemocytometer. Values represent the mean \pm S.D. of at least three determinations. B, the effects of C'-ARA54 and mt-ARA54 on AR transcriptional activity. LNCaP cells stably transfected with pBIG2i (vector alone), pBIG2i-C'-ARA54, pBIG2i-mt-ARA54, or pBIG2i-fl-ARA54 were transiently transfected with MMTV-Luc. After transfection, cells were cultured in the presence or absence of 2 μ g/ml doxy and 1 nM DHT as indicated. The Luc activity is presented relative to that in the absence of doxy and in the presence of DHT in each panel (black bars, set as 100%). Values represent the mean \pm S.D. of at least three determinations. C, the effects of C'-ARA54 and mt-ARA54 on PSA expression. Cell extracts from LNCaP cells stably transfected with pBIG2i (vector alone), pBIG2i-C'-ARA54, or pBIG2i-dn-mt-ARA54 cultured for 48 h with 1 nM DHT in the presence or absence of 2 μ g/ml doxy as indicated were analyzed on Western blots using an antibody to the PSA. The 33-kDa protein was detected as indicated and quantitated by Collage image analysis software (Fotodyne). The normalized expression level in the first lane (vector alone without doxy treatment) was set as 100%. Values represent the mean \pm S.D. of three separate experiments.

larly, a stronger 3-kb band was detected in the LNCaP cells stably transfected with fl-ARA54 when treated with doxy compared with no doxy treatment or transfection with vector (pBIG2i) alone.

Next, we tested the effect of the dominant-negative mutants of ARA54 on cell proliferation of the stable LNCaP cells, which had endogenous AR and wild-type ARA54. As shown in Fig. 4A, the expression of the mt-ARA54 (+ doxy) resulted in a significant decrease in cell growth. As a control, we also tested the effects of fl-ARA54 in LNCaP and mt-ARA54 in AR-negative

PC-3 cells. The results show that fl-ARA54 or mt-ARA54 without AR does not suppress prostate cancer cell growth. The Luc assay also demonstrated that using transient transfection of a reporter gene into these stable cell lines, the expression of the mt-ARA54 (+ doxy) significantly decreased AR transcriptional activity in the presence of DHT (Fig. 4B). These results confirm and strengthen our transient transfection data as described earlier.

The PSA is an AR target gene and presently the most useful marker to monitor the progression of prostate cancer. There-

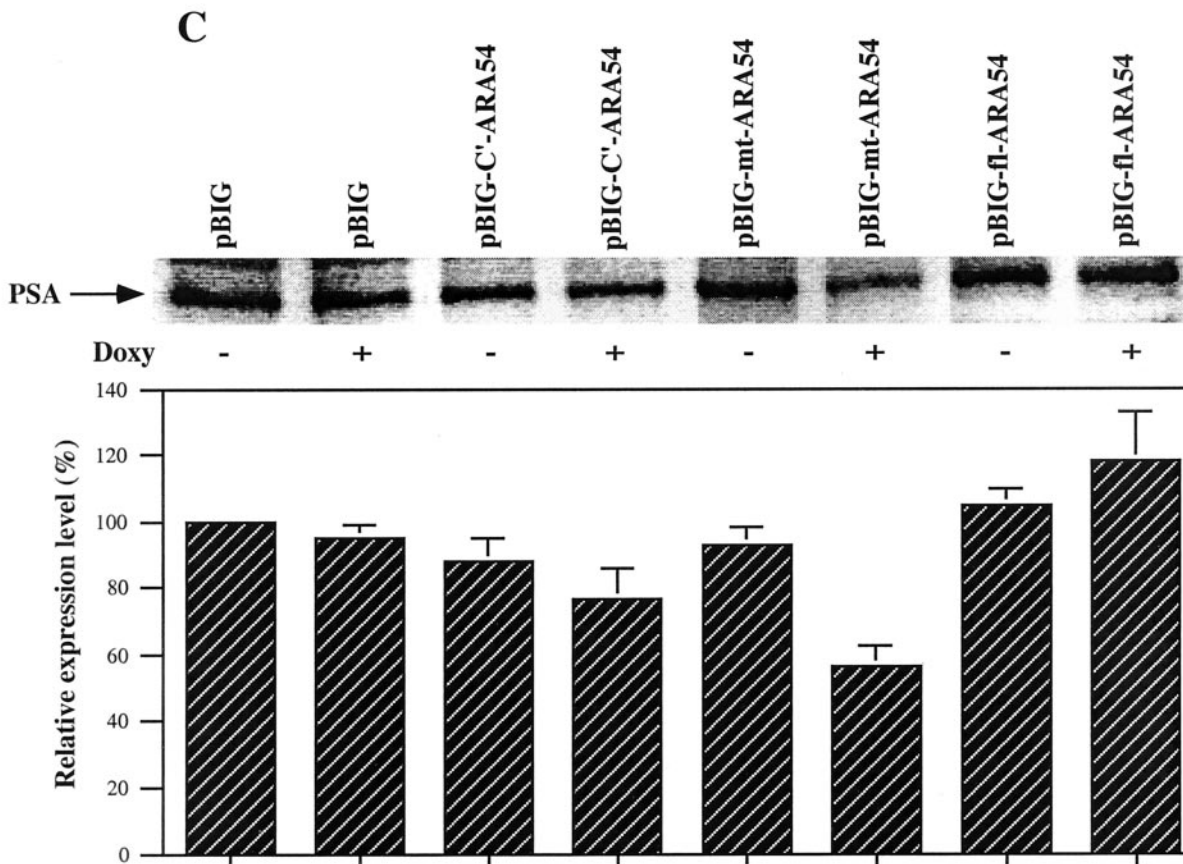
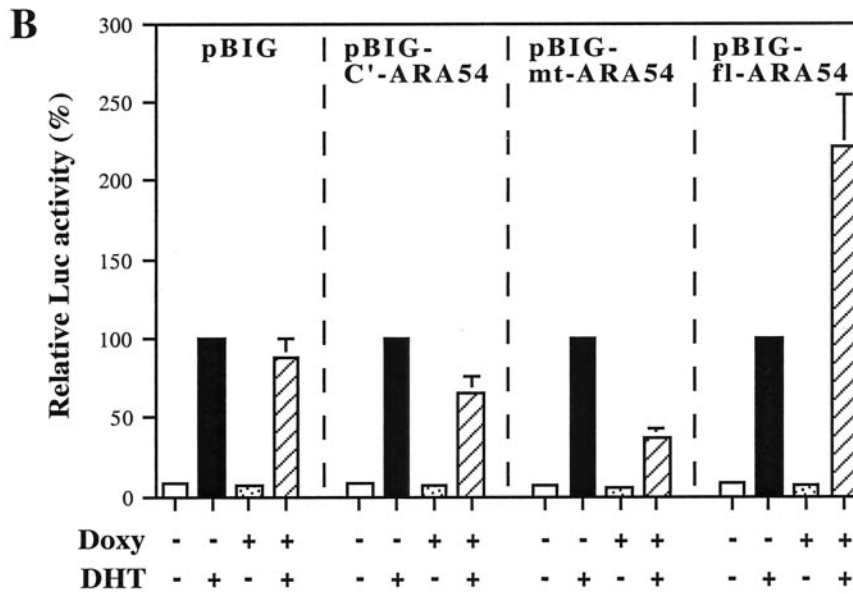


FIG. 4—continued

fore, it is of interest to determine whether the overexpression of the mutant ARAs as dominant-negative inhibitors of AR transcription suppresses PSA expression in prostate cancer cells. The Western blotting assay showed that endogenous PSA expression in the LNCaP cells was decreased to 60 and 87% when the mt-ARA54 and C'-ARA54 were expressed in the cells (+ doxy), respectively (Fig. 4C). There were no differences in AR protein levels in the LNCaP cells cultured with or without doxy (data not shown). These results suggest that a dominant-neg-

ative mutant ARA54 can inhibit AR-mediated prostate cancer progression.

Effect of the Dominant-Negative ARA54 Mutant on AR-ARA54 and ARA54-ARA54 Interactions—To investigate the potential mechanism through which mt-ARA54 suppresses ARA54-enhanced AR transactivation, we used a mammalian two-hybrid assay. DU145 cells were cotransfected with a GAL4 DBD and a VP16 activation domain fusion protein. Protein-protein interaction was assessed by measuring the activity of

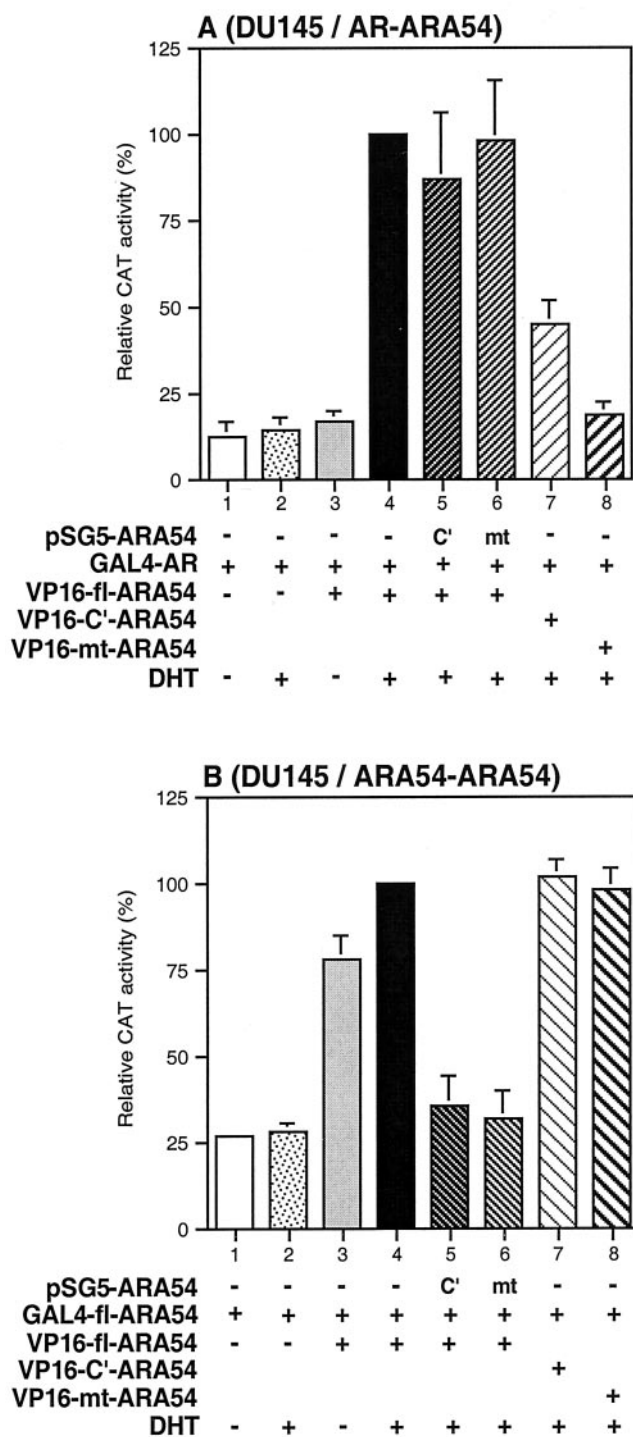


FIG. 5. The effects of C'-ARA54 and mt-ARA54 on AR-ARA54 and ARA54-ARA54 interactions. DU145 cells were transfected with 2.5 μ g of GAL4-hybrid expression plasmid pGAL0-AR (A) or pCMX-GAL4-DBD-fl-ARA54 (B), 2.5 μ g of VP16-hybrid expression plasmid pCMX-VP16-fl-ARA54, and 2.5 μ g of pG5-CAT with or without 2.5 μ g of pSG5-C'-ARA54 (C') or pSG5-mt-ARA54 (mt). pCMX-VP16-C'-ARA54 and pCMX-VP16-mt-ARA54 were also cotransfected to test the interactions with AR (A) and fl-ARA54 (B). The total amount of DNA was adjusted to 11.0 μ g with pSG5 and/or pVP16 for each transfection. The CAT activity was determined, and each CAT activity is presented relative to that of lane 4 in each panel (black bars, set as 100%). Values represent the mean \pm S.D. of at least three determinations.

the pG5-CAT reporter gene. First, we tested the influence of mt-ARA54 on the interaction between AR and fl-ARA54. As shown in Fig. 5A, AR interacted with fl-ARA54 in an androgen-dependent manner (lanes 1–4) as reported previously (26). The

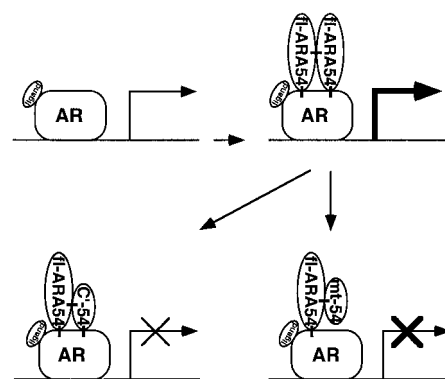


FIG. 6. Model for suppression of AR activity by C'-ARA54 and mt-ARA54. Fine and bold lines indicate the strength of transcription or inhibition. See text for detailed description.

addition of C'-ARA54 or mt-ARA54 resulted in very little change in AR-ARA54 interaction (lanes 5 and 6). Also, AR still interacted with C'-ARA54 but not with mt-ARA54 (lanes 7 and 8), consistent with our yeast two-hybrid screening results. We next tested whether ARA54 forms oligomers possibly in the dimeric form and the possible influence of mt-ARA54. As shown in Fig. 5B, GAL4-fl-ARA54 interacted with VP16-fl-ARA54 in the presence or absence of androgen (lanes 1–4), suggesting fl-ARA54 can form homodimers in an androgen-independent manner. When cotransfected with C'-ARA54 or mt-ARA54, CAT activities returned to the basal levels (lanes 5 and 6). Interestingly, fl-ARA54 can still interact with C'-ARA54 or mt-ARA54 (lanes 7 and 8). These results suggest that C'-ARA54 and mt-ARA54 may function in a dominant-negative manner through blocking the homodimerization of fl-ARA54.

DISCUSSION

In this study, we have identified a dominant-negative mutant of an AR coactivator, ARA54, using *in vitro* mutagenesis and a yeast two-hybrid screening assay. We generated a mutated C-terminal ARA54 library using hydroxylamine-mediated mutagenesis to induce random transition mutations (38). The mutant ARA54, mt-ARA54, carrying a glutamic acid to lysine substitution at codon 472 has lost its binding ability to AR and significantly suppressed the ability of endogenous or exogenous fl-ARA54 to enhance AR transcription in prostate cancer cells. The inhibitory effect was more obvious for exogenously expressed fl-ARA54 in DU145 cells than for endogenously expressed ARA54 in PC-3 and LNCaP cells. This result may be attributed to the presence of other AR coactivators, such as ARA70 and ARA55, which are slightly influenced by the mt-ARA54 in PC-3 and LNCaP cells. Importantly, although C'-ARA54 has been shown to have a weak dominant-negative effect, the mutant derived from this C-terminal fragment had a stronger suppressive effect on AR transactivation as well as on AR-mediated prostate cancer proliferation.

ARA54 has the ability to form homodimers as determined by using a mammalian two-hybrid assay. Because C'-ARA54 or mt-ARA54 did not influence fl-ARA54-AR interaction but did influence the interaction between fl-ARA54 and fl-ARA54, the molecular mechanism of these dominant-negative mutants appears to involve the formation of inactive dimers with fl-ARA54. In Fig. 6, we present a working model for the repression of AR transcriptional activity by C'-ARA54 or mt-ARA54. AR transactivation is induced by androgen and further enhanced through the interaction of AR with ARA54. For it to enhance AR transactivation, ARA54 may need to form homodimers. When fl-ARA54 dimerizes with C'-ARA54 or with mt-ARA54, the capacity of ARA54 to enhance transcription is reduced, resulting in a decrease in the observed AR-mediated

transactivation. It has been proposed that nuclear receptors may interact with a complex of coregulators involving coregulator-coregulator interactions in addition to coregulator-receptor interactions (39, 40). Dimerization between fl-ARA54 and mt-ARA54 may not be productive because of a reduced ability to interact with CBP or the basal transcriptional machinery.

Both normal prostate development and prostate cancer growth are largely dependent on the presence of androgens. Consequently, androgen ablation and/or blockage of androgen action through AR produces a brief response in most prostate cancer patients. However, in some cases prostate tumors are induced to proliferate by antiandrogens exerting an agonistic effect (5, 31), and androgen dependence is eventually lost during treatment (41). It has been suggested that because of changing the activity, *i.e.* altering ligand specificity by AR variations and abnormalities, the activation of AR pathway probably remains important in most prostate cancer cells from patients with clinically defined androgen-independent disease (42). Thus, in addition to current endocrine therapy, new approaches leading to the inhibition of AR-mediated prostate cancer growth are needed. Currently, several *in vivo* gene therapies involving the insertion of suicide genes, the replacement of mutated tumor suppressor genes, and antisense strategies are being evaluated in prostate cancer model systems as potential treatments (43). We here propose that the suppression of AR coactivator function may result in AR inactivation. Indeed, a recent report showed the physiological necessity of an AR coactivator. A loss of the function resulted in a complete androgen-insensitivity syndrome patient in whom the AR gene was completely normal (44). Because mt-ARA54 suppresses androgen-mediated and antiandrogen-mediated AR transactivation and PSA expression in prostate cancer cells, these results may lead to the development of new types of gene therapy strategies using mutant ARA54 or other suppressive mutant coactivators. For this strategy to be feasible, we first should clarify the expression levels of ARA54, the ratios of AR and ARA54 expression, and the mutations of ARA54 in prostate cancer tissues. Because the effect of mt-ARA54 was specific for ARA54-induced AR activity and that ARA54 is not the only coactivator to enhance AR activity, it may be necessary to identify dominant-negative mutants of other AR coactivators to obtain maximal AR suppression. Furthermore, antitumor activities of the dominant-negatives *in vivo* need to be evaluated.

In conclusion, it is suggested that ARA54 enhances AR transactivation by interacting with AR and dimerizing with itself. We found that a mutant form of the C-terminal fragment of ARA54 suppressed AR transactivation in a dominant-negative manner in prostate cancer cells by blocking the homodimerization of fl-ARA54. Its overexpression also inhibited AR-mediated prostate cancer proliferation. The mt-ARA54 thus represents a feasible new approach to inhibiting AR-/ARA54-mediated bioactivities. The use of dominant-negative coregulators such as mt-ARA54 may ultimately contribute to the control of AR-mediated prostate tumor progression.

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