Endocrine, *vol. 26, no. 1, 1–9, February 2005* 0969–711X/05/26:1–9/$30.00 © 2005 by Humana Press Inc. All rights of any nature whatsoever reserved.

Androgen-Receptor Coregulators Mediate the Suppressive Effect of Androgen Signals on Vitamin D Receptor Activity

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Overexpression of androgen receptors (AR) in PC-3 cell, and treatment of 5α-dihydrotestosterone in LNCaP cells lead to the suppression of VDR transactivation. Competition for shared coregulators between AR and VDR is one possible mechanism to explain the suppressive effect of androgen-AR signals on VDR activity. Among the AR coregulators we tested, ARA54, ARA70, supervillin, and gelsolin were found to enhance VDR transactivation. Further characterization of the interaction between ARA54 or ARA70 and VDR demonstrated a direct interaction between VDR and ARA70, but no association between ARA54 and VDR. The LXXLL motif of ARA70 is essential for interaction with VDR and partially responsible for its function as a coactivator of VDR. The suppression of VDR transactivation by AR signal was restored by overexpression of ARA70, but not ARA54. Together, ARA70 and ARA54 modulate VDR transactivation, and the competition for ARA70 mediates the suppressive effect of androgen-AR on VDR transactivation.

Key Words: 1α,25-Dihydroxyvitamin D_3; vitamin D_3 receptor; androgen receptor; coregulators.

Introduction

Treatment with 1α,25-dihydroxyvitamin D_3 (1,25-VD) inhibits proliferation and promotes differentiation in several types of cancers *(1)*. The implications for clinical usefulness has triggered a number of studies regarding the use of 1,25-VD to treat prostate cancer (PCa) *(2)*. Among several established human PCa cell lines, including LNCaP, DU145, PC-3, ALVA-3, and MDA PCa, treatment with 1,25-VD led to antiproliferation effects, but in varying degrees *(3,4)*. For example, DU145, PC-3, ALVA-3, and MDA PCa 2a display less growth inhibition with 1,25-VD (<20% inhibition) than LNCaP and MDA PCa 2b (approx 50%). These varied degrees of 1,25-VD growth inhibition indicate cells develop resistance to 1,25-VD treatment and hence become less sensitive to 1,25-VD. The receptor for 1,25-VD (VDR), which transmits the ligand signal to the nucleus and regulates the transcription of target genes, is universally expressed in the PCa cells studied *(3,4)*. The antiproliferation effect of 1,25-VD is suggested to be partly correlated with VDR expression level and transactivity *(3)*. Signals affecting VDR activity are potentially involved in the development of 1,25-VD resistance. The fact that 1,25-VD-resistant cells are also androgen-independent implies that the defects in 1,25-VD signaling may be a result of the altered molecular context derived in such forms of PCa.

Androgens are important for the growth of PCa, and antiandrogens have been used therapeutically for decades. Pharmacological or surgical androgen ablation therapy is commonly used to treat PCa patients. Although tumors shrink significantly after therapy, the majority of patients develop hormone-refractory PCa. Numerous mechanisms revealed to date demonstrate that diverse signaling pathways are involved in developing resistance to androgen ablation *(5)*. First, mutant ARs with broad ligand sensitivity have been found to utilize hormones other than androgen to transmit growth-promoting signals *(5)*. Second, amplification of growth hormone signals, such as HER2/neu, in PCa can stimulate growth and eventually bypass AR *(5)*. Third, overexpression of coregulators, such as SRC-1, TIF-2, and gelsolin, in androgen-independent PCa has been shown, that may magnify AR activity in the presence of trace amounts of androgen *(6,7)*. A more recent finding also demonstrated that hydroxyflutamide, an antiandrogen, could activate the MAPK pathway, an effect that might contribute to the development of PCa resistance to androgen ablation *(8)*. Either one of mechanisms or a combination of several mechanisms described above can result in the development of androgen-independent cell growth, and therefore play roles in 1,25-VD resistance.

Most coregulators associate with and modulate more than one steroid receptor. AR coregulators, originally identified as AR-associated proteins, also modulate many other steroid receptors. As previously reported, ARA54 enhances AR and progesterone receptor *(9)*; ARA70 enhances peroxisome

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Received November 8, 2004; Revised December 15, 2004; Accepted December 16, 2004.

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proliferator activated receptor γ and AR *(10)*; and gelsolin and supervillin enhance AR and glucocorticoid receptor *(7,11)*. It is highly possible that VDR and AR share the same coregulators and the potential cross-talk between VDR and androgen-AR signals mediated by coregulators might account for vitamin D action in PCa. Therefore, evaluation of the function of androgen-AR signals and AR coregulators on VDR activity is the first step to elucidate their roles in vitamin D response. Here we characterize the effects of androgen-AR signals and AR coregulators on VDR transactivity, which may provide us information about potential mechanisms of 1,25-VD resistance in PCa cells.

Results

Androgen Signaling Suppresses VDR Transactivation

We tested how androgen-AR signals influence 1,25-VD signaling through VDR. The 1,25-VD–induced VDR activity is measured by the activation of VDR target gene, rat CYP24 promoter-controlled reporter gene, *rCYP24-LUC*. Overexpression of AR in PC-3 suppresses the 1,25-VD–induced VDR transactivation (Fig. 1A, lane 2 vs 5), with further suppression after addition of the androgen, 5α-dihydrotestosterone (DHT) (lane 5 vs 6). On the other hand, DHT treatment in the AR-containing cells, LNCaP, suppresses VDR activity (Fig. 1B). To examine whether the VDR amount was reduced after DHT treatment, we compared the VDR protein levels. As shown in Fig. 1C, the protein level of VDR was increased under the treatment of DHT (lane 3 vs lane 2). Therefore, the suppression of VDR activity by androgen-AR signals is not due to reduced amount of VDR under DHT treatment. The overexpression of AR in PC-3 and activation of AR in LNCaP, both suppress VDR transactivation, suggesting a possible cross-talk between AR and VDR in gene regulation.

Some of AR Coregulators Modulate VDR Transactivation

To investigate the cross-talk between AR and VDR signaling, we hypothesize that coregulators shared by VDR and AR may mediate the cross-talk between 1,25-VD and androgen signaling pathways. We have screened several known AR coregulators for their ability to modulate VDR transactivation. The COS-1 cell line was selected for examination of coregulators' effect on VDR due to the absence of most steroid receptors, which eliminates interference from other steroid receptors. Among several AR coregulators examined, ARA54, ARA70, gelsolin, and supervillin were found to enhance VDR (Fig. 2), whereas ARA55 and ARA24 had no effect on 1,25-VD–induced VDR transactivation (data not shown). SRC-1 and SMRT, the known VDR coactivator and corepressor, respectively, served as controls.

The relative expression levels of coregulators and VDR may affect coregulator function. By defining the minimum and maximum activity of coregulators, we can determine

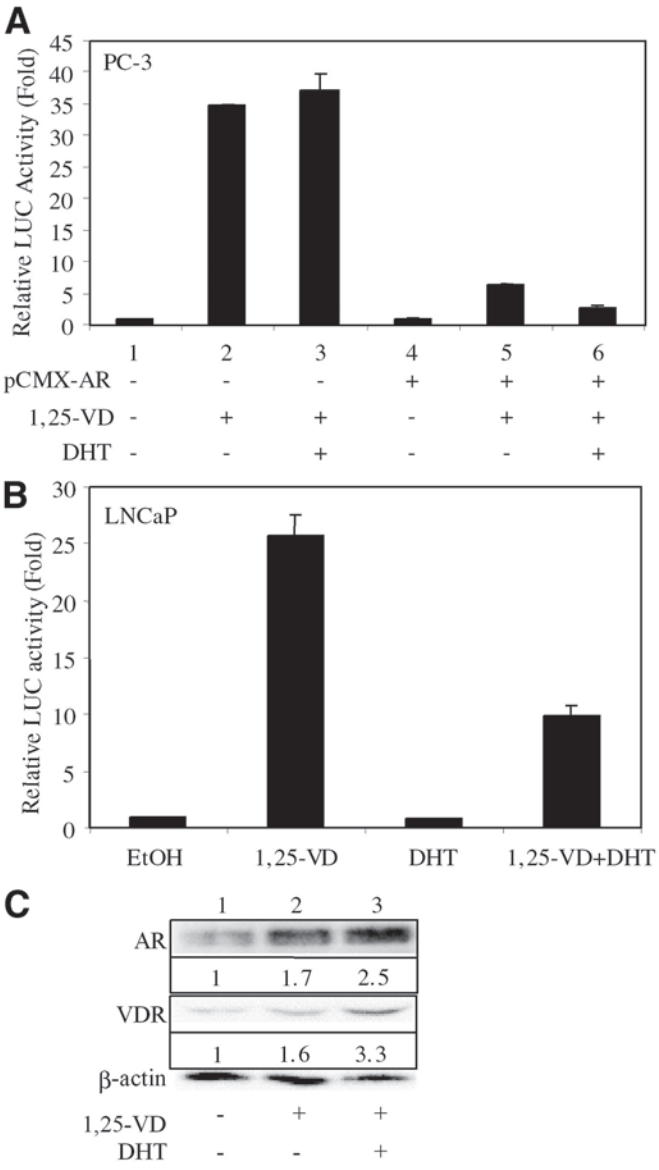

Fig. 1. Modulation of VDR transactivity by androgen-AR signal. (**A**) PC-3 cells were plated at a density of 10^5 cells/well in 24-well plate. Cells were co-transfected with 0.375 μg pCMV or pCMV-AR, 0.125 μg prCYP24-LUC, and 1 ng pRL-SV40 by SuperFect. After 20 h, cells were treated with EtOH or 10^{-7} *M* 1,25-VD for another 24 h before being harvested. The LUC activity relative to lane 1 was calculated and the mean ± SD of three independent experiments is shown. (**B**) LNCaP cells were plated at a density of 3×10^4 cells/well in 24-well plates. After 24 h, cells were transfected with 0.6 μg prCYP24-LUC and 1 ng pRL-SV40 by SuperFect. After 20 h, cells were treated with EtOH, 1,25-VD or DHT, as indicated, for another 24 h. Cells were then harvested for the LUC assay. The LUC activity relative to lane 1 was calculated and the mean ± SD of three independent experiments is shown. (**C**) The expression of AR and VDR after treatment in LNCaP cells was detected by Western blotting. LNCaP was seeded at a density of 10^6 cells/dish in 100 mm dishes. After treating with ligands as indicated for 24 h, cell lysates were harvested for the detection of AR, VDR, and actin expression amount. The level of expression was extrapolated by densitometric analysis after correction by actin amount.

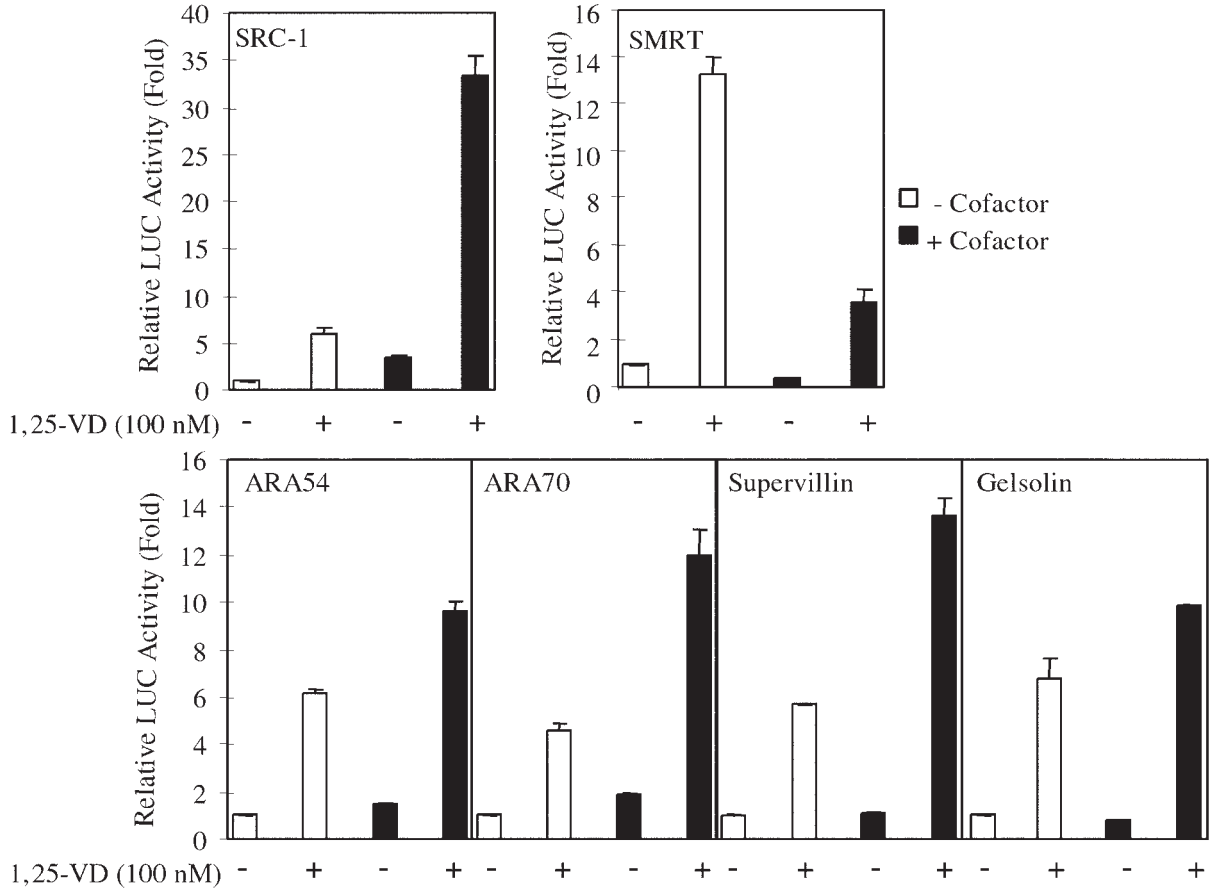

Fig. 2. The effects of coregulators on VDR transcriptional activity in COS-1 cells. COS-1 cells were plated at a density of 3×10^4 cells/well in 24-well plates, 24 h later cells were co-transfected with pSG5-AR or 0.2 μg pSG5-VDR, 0.6 μg coregulator expression vector or empty vector, and 0.2 μg prCYP24-LUC by SuperFect. After transfection overnight, cells were then incubated with either EtOH or 10^{-7} *M* 1,25-VD. After 24 h, cell lysates were prepared and used for LUC assay. The LUC activity relative to lane 1 was calculated and the mean ± SD of three independent experiments is shown.

coregulator efficiency and the influence of cellular context on coregulator function. Therefore, we titrated the relative coregulator plasmid compared with that of VDR from 1 to 1 up to 8 to 1. SRC-1 was found to enhance VDR and SMRT was found to repress VDR activity starting at a ratio of 1 to 1, while most AR coregulators tested were found to enhance VDR starting at a ratio of 4 to 1 (Fig. 3). Among AR coregulators, ARA70 was shown to be the strongest enhancer of VDR activity, whereas gelsolin and ARA54 showed the weakest enhancement of VDR transactivity in COS-1 cells. Therefore, we focused on ARA70 and ARA54 to further dissect their mechanisms of actions.

ARA70 Directly Associates with VDR But Not ARA54

Most coregulators associate with nuclear receptors and then modulate receptor activity. We determined whether ARA54 and ARA70N associated with VDR using the mammalian two-hybrid and GST pull-down assays. Some proteins may lose their function when conjugated with Gal4 (DBD) or VP16. Therefore, we selected the one with proper function maintained after conjugation with Gal4(DBD) or VP16 for mammalian two-hybrid assay. To ensure these conjugated coregulators have proper function, we demonstrated that Gal-ARA54C can interact with VP16-AR; VP16-VDR can interact with Gal-RXRα-LBD; and the VP16-ARA70N interacts with Gal-AR, which were used as positive controls (Fig. 4A). Co-transfection of plasmids expressing Gal-ARA54C with VP16-VDR demonstrated minor interaction between VDR and ARA54C (Fig. 4A). On the other hand, Gal-VDR interacted with VP16-ARA70N in a ligand-dependent manner in the two-hybrid system (Fig. 4A). We further demonstrated the interaction by using in vitro GST pull-down assay, which showed ligand-independent interaction between ARA70 and VDR-L (Fig. 4B), whereas there was no interaction between ARA54 and VDR-L. The ligand-independent interaction between ARA70 and VDR-L in GST-pull down assay while ligand-dependent interaction between ARA70N and VDR in mammalian two-hybrid assay implied another interaction domain may be located in the C-terminus of ARA70 that is responsible for such interaction. It may also result from fusion protein characteristics, or differences in the overall assay environments.

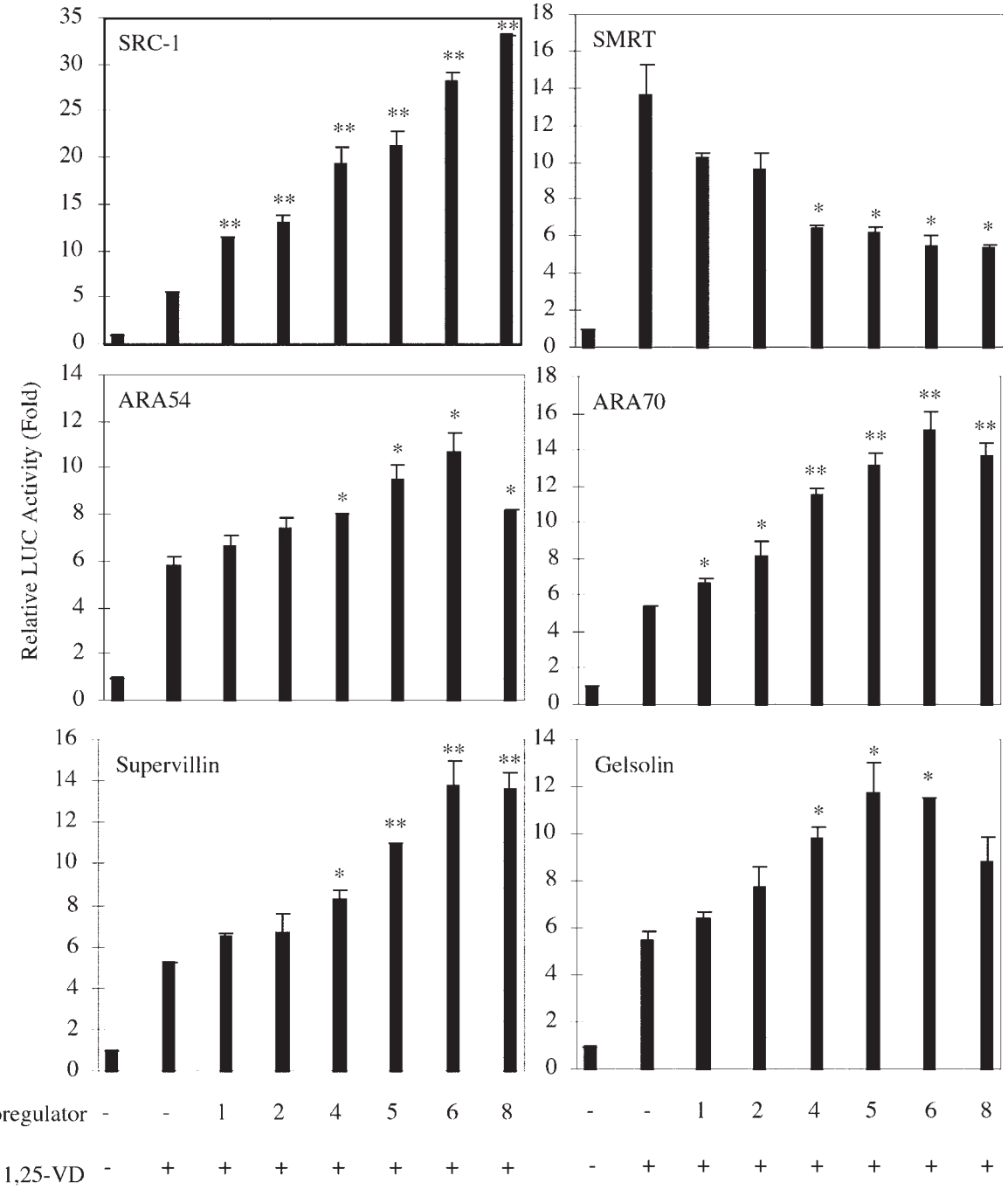

Fig. 3. The titration of coregulators shows dose-dependent effects in promoting VDR transcriptional activity. COS-1 cells were plated and transfected as described in Fig. 2 except that relative amounts of plasmids encoding coregulators to plasmids encoding VDR ranging from 1 to 1 up to 8 to 1 were transfected. The total amount of transfected plasmids was adjusted to 1 μg/well by addition of the empty vector. After transfection overnight cells were then incubated with EtOH or 10^{-7} *M* 1,25-VD. After 24 h, cell lysates were prepared and used for LUC assay. The LUC activity relative to lane 1 was calculated and the mean ± SD of three independent experiments is shown. *$p < 0.05$; **$p < 0.01$ significant differences compared with 1,25-VD treated groups.

LXXLL Motif in ARA70 Is Important for Interaction with VDR

LXXLL sequence motifs are important for most coregulators, including SRC-1, TIF-II, and NCoR, to associate with steroid receptors, such as estrogen receptor, RXR, and VDR. There is one LXXLL motif in ARA70 located in its N-terminus. Point-mutated ARA70N containing LXXAA instead of LXXLL was constructed and characterized for its interaction ability with VDR. By using mammalian two-hybrid assay, we demonstrated that VP16-ARA70N mutant (LXXAA) lost the interaction with Gal4(DBD)-VDR indicating the LXXLL motif in ARA70N is essential for interaction with VDR (Fig. 5A). We further tested the ability of mutant ARA70 to promote VDR transactivity. Overexpres-

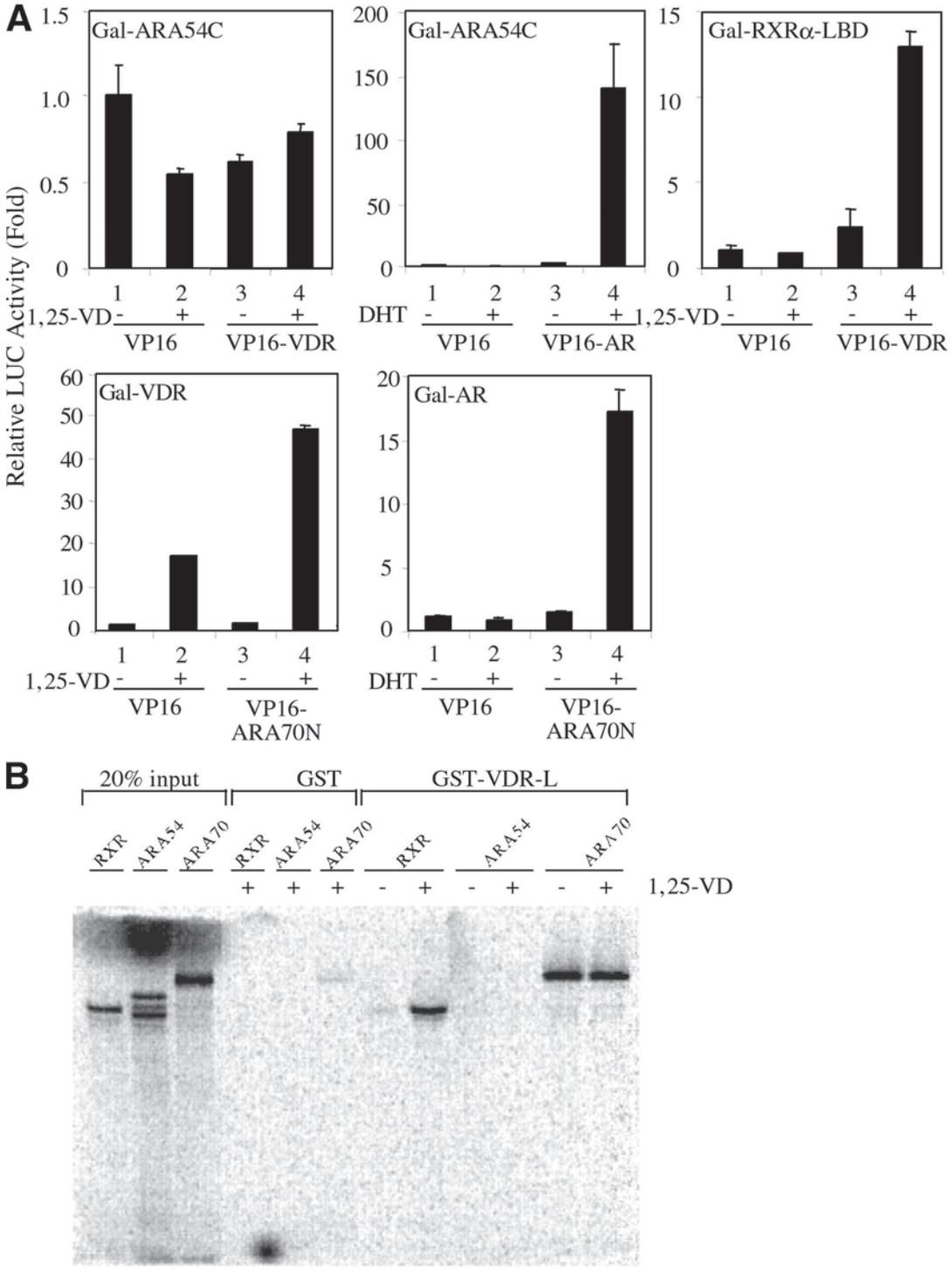

Fig. 4. The interaction between VDR and ARAs was analyzed in mammalian two-hybrid and GST pull-down assays. (**A**) COS-1 cells were plated at a density of 3×10^4 cells/well in 24-well plates. After 24 h, the mammalian two-hybrid assay was performed by co-transfection of plasmids expressing 0.2 μg Gal-VDR, -RXRα-LBD, or -ARAs, 0.2 μg VP16, VP16-VDR, -AR or -ARAs, 0.1 μg pG5-LUC, and 1 ng pRL-SV40 as indicated. After 20 h, cells were treated with EtOH, 10^{-8} *M* DHT, or 10^{-7} *M* 1,25-VD for another 24 h, and were then harvested for the LUC assay. The LUC activity relative to lane 1 was calculated and the mean ± SD of three independent experiments is shown. (**B**) GST and GST-VDR-L proteins were expressed and purified from bacteria. Full-length RXR, ARA54, and ARA70 were expressed in vitro and labeled with [^{35}S]methionine. After incubating with EtOH or 10^{-6} *M* 1,25-VD for 1 h at 4°C, GST-VDR-L was further incubated with RXR, ARA54, or ARA70 for another 2 h. The pull-down complexes were separated by 12% SDS-PAGE and analyzed by phosphorimager for the detection of radioactive signals.

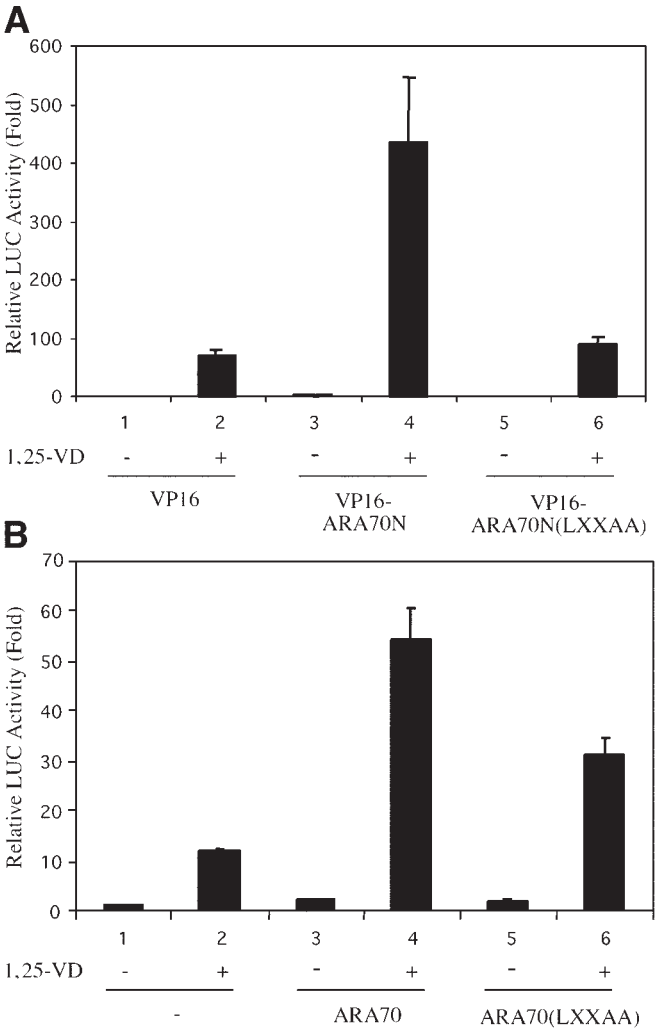

Fig. 5. The interaction and coactivator activity of ARA70 mutant to VDR were analyzed in mammalian two-hybrid and reporter assays. (**A**) COS-1 cells were plated at a density of 3×10^4 cells/well in 24-well plates. After 24 h, the mammalian two-hybrid assay was performed by co-transfection of plasmids expressing 0.2 μg Gal-VDR, with 0.2 μg VP16, VP16-ARA70, or VP16-ARA70(LXXAA), with 0.1 μg pG5-LUC, and 2.5 ng phRL-tk. After 20 h, cells were treated with EtOH or 10^{-7} *M* 1,25-VD for another 24 h, and were then harvested for the LUC assay. The LUC activity relative to lane 1 was calculated and the mean ± SD of three independent experiments is shown. (**B**) COS-1 were plated and transfected with 62.5 ng pSG5-VDR, 125 ng prCYP24-LUC, 2.5 ng phRL-tk, and 312.5 ng pSG5, pSG5-ARA70, or pSG5-ARA70(LXXAA). Cells were then treated and harvested for LUC assay as described in **A**.

sion of mutated ARA70 containing LXXAA motif in vitamin D–responsive reporter gene assay showed that coactivation of VDR was significantly reduced compared to wild-type ARA70 (Fig. 5B). However, the mutant ARA70 did not completely lose its coactivation function, which implies another interaction domain is located in the C-terminus of ARA70 that may still interact with VDR. These results indicate that the LXXLL motif in ARA70 is important for interaction with VDR and for co-activation of VDR.

Overexpression of ARA70 Restores VDR Transactivity Suppressed by Androgen

The fact that both VDR and AR can interact with ARA70 indicates the cross-talk between these two receptors may be mediated by competing for ARA70. We tested whether we could overexpress sufficient amounts of ARA70 for VDR transactivation even in the presence of androgen. Different amounts of plasmids encoding ARA70 protein were transfected into LNCaP where androgen treatment suppressed VDR transactivity (Fig. 1B). The androgen-suppressed VDR transactivity was dose-dependently restored by increasing the transfected amount of ARA70 plasmids (Fig. 6). However, ARA54, which promoted VDR through an indirect mechanism, could not restore VDR transactivity suppressed by androgen. Therefore, androgen treatment did not prevent ARA54 from promoting VDR transactivity, but did squelch ARA70 from interacting with VDR, hence decreasing VDR transactivity.

Discussion

The finding that androgen-AR signal can attenuate VDR transactivation led us to investigate whether AR coregulators can interact with VDR and enhance VDR transactivation. The interaction between AR coregulators and VDR suggests the cross-talk between AR and VDR is mediated by shared coregulators. The interaction between ARA70 and VDR implies that AR and VDR could be linked together by ARA70, or that they may compete for binding to ARA70. Most coregulators utilize the LXXLL sequence motifs to associate with steroid receptors, while AR coregulators also utilize a unique motif, FXXLF *(12,13)*. Interestingly, these two motifs co-exist in ARA70, implying that AR and another steroid receptor may associate with ARA70 without competing with each other. Further investigation showed that VDR associates with ARA70 through the LXXLL motif, but not the FXXLF motif (data not shown). Our result showing that VDR transactivity suppressed by androgen was restored by overexpression of ARA70 indicates that the competition for association with ARA70 between VDR and AR mediates this cross-talk. Another AR coregulator, ARA54, also enhances VDR transactivity, but no direct interaction between these two proteins was observed in either the mammalian two-hybrid or GST pull-down assay. A recent study has shown that ARA54 may act as a Ub-ligase (E3) and exert proteasome function *(14)*. Therefore, the degradation of VDR partners may be regulated by ARA54, an effect that indirectly modulates VDR transactivity. This mechanism may exist in spite of no direct interaction with VDR. Because androgen signals did not squelch ARA54 from promoting VDR transactivity, the overexpression of ARA54 could not restore VDR transactivity in the presence of androgen.

This observation also suggests that the context of AR and AR coregulators in cells can contribute to how androgen-AR affects the vitamin D-VDR signaling. Among all the co-

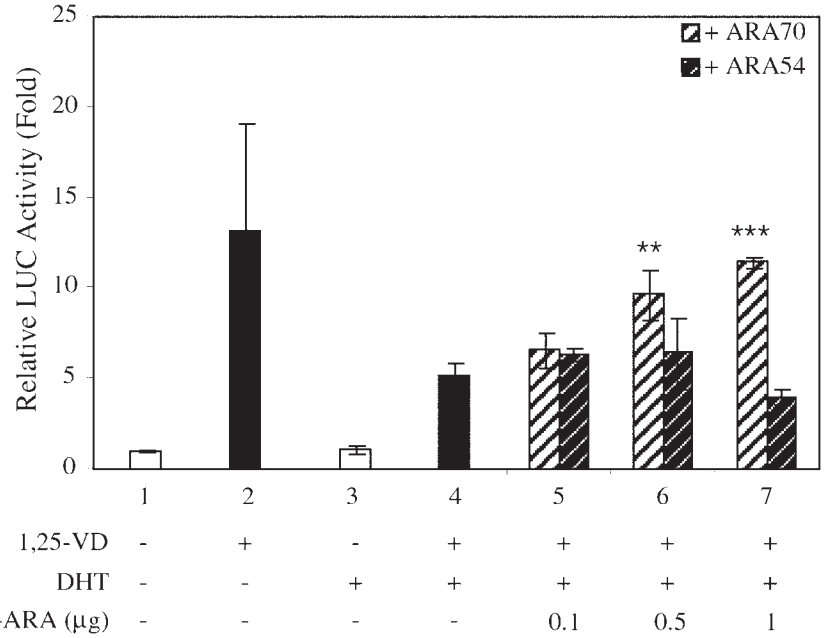

Fig. 6. The dose-dependent effects of ARA70 in restoring VDR transcriptional activity suppressed by androgen signal. LNCaP cells were plated as described in Fig. 1. The amount of plasmids encoding coregulators ranging from 0.1, 0.5, to 1 μg were transfected. Total amount of transfected plasmids was adjusted to 1 μg/well by empty vector. After 2–3 h incubation, cells were allowed to recover with CD-FBS supplemented medium overnight. Cells were then incubated with EtOH, 10^{-7} *M* 1,25-VD or/and 10^{-8} *M* DHT. After 24 h, cell lysates were prepared and used for LUC assay. The LUC activity relative to lane 1 was calculated and the mean ± SD of three independent experiments is shown. $^{**}p < 0.01$; $^{***}p < 0.005$ significant differences compared to lane 4.

activators we tested, only some show the enhancing effect. In fact, ARA55 did not promote VDR transactivation (data not shown). The order of promoting capacity is SRC-1 > ARA70 > supervillin > gelsolin > ARA54 (Fig. 3). Some studies report the overexpression of coregulators, including SRC-1 and gelsolin, in hormone-refractory PCa. This implies enhanced VDR transactivity, hence the reponse to vitamin D may occur in hormone-refractory PCa. More studies are needed to characterize the expression levels of other coregulators in various stages of PCa. Nonetheless, one report demonstrated that 31% of hormone-refractory tumors contained AR gene amplification *(15)*. Increased expression of AR during PCa progression may result in suppressed VDR activity and diminished antiproliferation effects of vitamin D in higher-grade PCa patients. The composition of AR and coregulators in cells provides an environment where VDR activity is affected. Therefore, when evaluating the antiproliferation effect of vitamin D in prostate cancer, the impact from overexpression of AR and coregulators should both be considered.

Several studies demonstrate the antiproliferation effect of vitamin D in androgen-responsive PCa LNCaP cells is dependent on functional AR. First, the expression of AR is induced by 1,25-VD and this induction correlates with growth inhibition effect of 1,25-VD *(16)*. Second, anti-androgen treatment attenuates the growth-inhibitory effect of 1,25-VD in LNCaP cells *(16)*. Third, AR RNAi depleted AR expression results in less growth inhibitory effect of 1,25-VD *(17)*. However, antiandrogen did not affect the anti-proliferation effect of 1,25-VD in androgen-refractory PCa cell lines, MDA PCa, and LNCaP-104R1 *(4,18)*. Overexpression of AR in ALVA 31 did not restore the response to 1,25-VD *(18)*. Those results indicate that 1,25-VD no longer depends on AR signaling for growth inhibition in these cell lines. The dilemma that 1,25-VD inhibits PCa growth through functional AR while functional AR suppress VDR transactivity complicates the outcome from the cross-talk between VDR and AR. Therefore, whether the functional AR affected VDR transactivity can be translated into the antiproliferation effect of 1,25-VD may depend on how cells rely on androgen-AR signal for growth.

In addition to PCa cells, VDR is widely expressed and functions in many tissues, such as parathyroid gland, bone, skin, and intestine *(19)*. The fact that AR coregulators modulate VDR suggests that AR can suppress VDR activity by competing for the same coregulators in common target tissues of VDR and AR, such as the hair follicle and bone. Androgenetic alopecia occurring in men is androgen-dependent, and androgen-regulated factors secreted from dermal papilla cells are believed to inhibit the growth of components of the hair follicle *(20)*. Because alopecia also occurs in VDR knockout mice, the androgenetic alopecia might result from the suppression of VDR activity by androgen signals in the hair follicle *(21)*. In addition, the bone volume

in AR knockout mice (ARKO) is reduced, and osteopenia may result from increasing numbers of osteoclasts *(22)*. The formation of osteoclasts is induced by active VDR *(23)*, which implies a potential mechanism for the stimulation of osteoclasts in ARKO mice. The physiological significance of AR signaling on VDR target genes and the roles of coregulators in mediating this cross-talk should be further investigated.

In summary, the fact that overexpression of AR in PC-3 cells and treatment of LNCaP cells with DHT both suppress endogenous VDR transactivation, supports the existence of cross-talk between VDR and AR in gene regulation. To investigate the underlying mechanism, we demonstrated that several AR coregulators, including ARA54, ARA70, gelsolin, and supervillin, all promote VDR. Furthermore, ARA70, but not ARA54, associates with VDR and overexpression of ARA70 can restore the androgen-AR signal suppressed VDR transactivity. Therefore, the cross-talk between VDR and AR is through sharing same coregulators. The altered composition of AR and AR coregulators, which frequently occurs during PCa progression, may contribute to 1,25-VD resistance in PCa. This cross-talk could possibly exist in other tissues and further studies are required to elucidate the physiological significance resulting from the interplay between androgen signal, androgen coregulators, and 1,25-VD signals.

Materials and Methods

Plasmids

prCYP24-LUC was constructed by inserting the fragment containing –950 bp–−55 bp region of rCYP24 promoter released from rat 25-hydroxyvitamin D_3 24-hydroxylase gene promoter CAT construct (–2.2 kb–+188 bp), a kind gift from Dr. Yoshihiko Ohyama (Hiroshima University, Japan), by MscI and SacI into SacI and SmaI sites of pGL3-TK (a gift from Dr. Eungseok Kim, University of Rochester, Rochester, NY). The cDNA encoding full-length VDR was amplified by PCR and inserted into the pCMX-VP16 vector and pCMX-Gal4.N vector to generate the VP16-VDR and Gal4-VDR expression plasmids. pCMV-Gal4-ARA54C (amino acids 361–474), pCMV-Gal4-retinoid x receptor α (RXRα)–ligand binding domain (LBD), and pG4AB1-hAR(DE) (amino acids 624–918) were used for expression of Gal4-DNA binding domain (DBD)–conjugated proteins. The plasmids pCMX-VP16-ARA70N (amino acids 1–401) and pCMX-VP16-AR were used to express VP16-conjugated ARA70N and AR full-length proteins. The cDNA encoding the LBD of VDR (VDR-L) was amplified by PCR and inserted into the pGEX-KG vector to generate the GST-VDR-L expression plasmid. The plasmid expressing VP16-conjugated mutant ARA70N (LXXAA) for mammalian two-hybrid, pCMX-VP16-ARA70N(LX XAA), was described previously *(24)*. pSG5-ARA70(LX XAA) was constructed by replacing a fragment from *Eco*RI and *Bgl*II digested pSG5-ARA70 with a fragment released from EcoRI and *Bgl*II digested pCMX-VP16-mtARA70.

Transfection Studies

LNCaP and PC-3 cells were maintained in RPMI-1640 containing 10% FBS. COS-1 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% FBS. Cells were seeded 24 h before transfection, then transfected according to the "SuperFect Transfection" instructions (Qiagen, Valencia, CA). After 2–3 h incubation, culture medium was replaced with medium supplemented with 10% charcoal-dextran treated FBS (CD-FBS). After 20 h, cells were treated with EtOH, 1,25-VD, or DHT, as indicated, then further incubated at 37°C for 16–24 h before harvesting. Cell lysates were prepared and used for the luciferase (LUC) assay according to the manufacturer's instructions (Promega, Madison, WI). LUC activity was normalized with internal control *Renilla*-LUC activity. The results were obtained from at least three independent experiments and presented as means ± SD.

Mammalian Two-Hybrid Assay

COS-1 cells were plated at a density of 3×10^4 cells/well in 24-well plates. After 24 h, the mammalian two-hybrid assay was performed by co-transfection of plasmids expressing 0.2 μg Gal4(DBD)-conjugated receptors or ARAs, 0.2 μg VP16 alone, VP16 conjugated receptors, or ARAs, 0.1 μg pG5-LUC, and 1 ng pRL-SV40. After 20 h, cells were treated with EtOH, 10^{-8} M DHT, or 10^{-7} *M* 1,25-VD for another 24 h, and were then harvested for the LUC assay. The LUC activity represents interaction between receptors and coregulators tested.

Glutathione S-Transferase (GST) Pull-Down Assay

GST-VDR-L fusion protein, and GST control protein were purified *(25)*. The GST fusion proteins were pulled down by glutathione (GSH) beads at 4°C for 1 h and washed three times with washing buffer. The purified GST fusion proteins and beads were incubated in 100 μL binding buffer with EtOH or 1 μ*M* 1,25-VD for 1 h, at 4°C. In vitro-translated ^{35}S-methionine-labeled RXR, ARA54, and ARA70 full-length proteins were produced using the TNT-coupled reticulocyte lysate system (Promega, Madison, WI). Five microliters of RXR, ARA54, or ARA70 was then added. After incubating for 2 h at 4°C, GSH beads were washed with washing buffer three times. Protein complexes were separated by 12% SDS-PAGE and visualized by Phosphor Imager (Molecular Dynamics Amersham Bioscience, Piscataway, NJ).

Preparation of Cellular Protein and Western Blots

LNCaP cells were seeded in 100-mm dishes and treated with ligands. After 24 h, cells were collected and suspended in lysis buffer, and centrifuged. Aliquots corresponding to

100 μg protein of each sample were loaded onto a 8% SDS-PAGE for Western blotting. After blotting, the membrane was blocked with 5% nonfat milk and probed with antibody against AR (NH27), VDR (Santa Cruz Biotechnology, Santa Cruz, CA), and actin (Santa Cruz Biotechnology) at a dilution of 1:2000 in PBS containing 0.1% Tween 20, for 1 h. After washing, the membrane was incubated with the alkaline phosphatase–conjugated anti-rabbit antibody (Santa Cruz Biotechnology) for 45 min, washed again, and the immunoreactive bands were visualized for alkaline phosphatase activity with the 5-bromo-4-chloro-3-indolylphosphate-nitro blue tetrazolium phosphatase substrate (Bio-Rad Laboratories, Hercules, CA).

Acknowledgments

Expression plasmids of ARA54, ARA70, supervillin, and gelsolin were kindly provided by Dr. Chawnshang Chang (University of Rochester). This work was supported by the Department of Defense grant PC040630.

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