

Androgen Receptor (AR) NH₂- and COOH-Terminal Interactions Result in the Differential Influences on the AR-Mediated Transactivation and Cell Growth

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Early reports showed that androgen receptor (AR) NH₂- and COOH-terminal (N-C) interaction was important for full AR function. However, the influence of these interactions on the AR *in vivo* effects remains unclear. Here we tested some AR-associated peptides and coregulators to determine their influences on AR N-C interaction, AR transactivation, and AR coregulator function. The results showed that AR coactivators such as ARA70N, gelsolin, ARA54, and SRC-1 can enhance AR transactivation but showed differential influences on the N-C interaction. In contrast, AR corepressors ARA67 and Rad9 can suppress AR transactivation, with ARA67 enhancing and Rad9 suppressing AR N-C interaction. Furthermore, liganded AR C terminus-associated peptides can block AR N-C in-

teraction, but only selective peptides can block AR transactivation and coregulator function. We found all the tested peptides can suppress prostate cancer LNCaP cell growth at different levels in the presence of 5 α -dihydrotestosterone, but only the tested FXXLF-containing peptides, not FXXMF-containing peptides, can suppress prostate cancer CWR22R cell growth. Together, these results suggest that the effects of AR N-C interactions may not always correlate with similar effects on AR-mediated transactivation and/or AR-mediated cell growth. Therefore, drugs designed by targeting AR N-C interaction as a therapeutic intervention for prostate cancer treatment may face unpredictable *in vivo* effects. (*Molecular Endocrinology* 19: 350-361, 2005)

ANDROGEN-ANDROGEN RECEPTOR (AR) mediates a wide spectrum of physiological and developmental states and is important in prostate cancer progression (1-3). AR is a transcriptional factor that belongs to the nuclear receptor superfamily (4-7), containing a conserved NH₂- (N-) terminal functional domain, a DNA binding domain (DBD), a hinge region, and a COOH- (C-) terminal ligand binding domain (LBD) (8, 9). Before binding to its natural ligands, tes-

tosterone and 5 α -dihydrotestosterone (DHT), AR may form a complex with chaperones such as heat shock proteins 70 and 90 that are located mainly in the cytosol (10). After ligand binding, AR dissociates from chaperones, phosphorylates, and translocates into the nucleus. AR then binds to DNA response elements on target gene promoters as a dimer and recruits some selective type I coregulators to enhance target gene transcription (11, 12).

NH₂- and COOH-termini (N-C) on the AR can bind to each other in the presence of a ligand, which may play an important role for full AR function via influencing dimerization, slowing down ligand dissociation from the LBD, and AR degradation (13, 14). Disruption of AR N-C interactions has been linked to androgen-insensitive syndrome (15, 16). These N-C interactions can be a tool to differentiate between androgens and antiandrogens (17). The activation function-2 (AF-2) in the C terminus of nuclear receptors is important for recruiting LXXLL (where L is leucine and X is any amino acid) motifs in coregulators to assist transcription (17-19). Two LXXLL-like (F/W)XXLF (where F is phenylalanine, L is leucine, W is tryptophan, and X is any amino

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Abbreviations: AF-2, Activation function-2; AR, androgen receptor; ARA, AR-associated protein; DBD, DNA binding domain; DHT, 5 α -dihydrotestosterone; F, phenylalanine; H, histidine; L, leucine; LBD, ligand binding domain; LUC, luciferase; M, methionine; MMTV, mouse mammary tumor virus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N-C, NH₂-COOH-terminal; PSA, prostate-specific antigen; SV40, simian virus 40; TAT, fragment of transactivating regulatory protein of human immunodeficiency virus type 1.

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acid) motifs, which are important for AR N-C interactions, exist in the AR N terminus. The FXXLF motif of AR can compete with the LXXLL motif of a coregulator to bind to the AR AF-2 hydrophobic groove (20, 21). This FXXLF motif can also influence AR-heat shock protein 90 interaction but will not influence AR ligand binding or interference between AR and DNA response elements (22). In AR-associated proteins such as ARA70 (23), ARA55 (24), ARA54 (25), FHL2 (26), and Rad9 (27), the FXXLF motif is important in coregulator functions, and could mediate interactions between AR and those coregulators. Using phage display techniques, we also found that (F/W)XXL(F/W) and FXXLY motifs exist in AR C terminus associated proteins (22). Electrostatic modulation around the FXXLF motif and hydrophobic forces in the C-terminal of the motif can assist peptide binding to the AF-2 of liganded AR (22, 28). Some short peptides containing these motifs can block AR N-C interactions and selective peptides can block AR transactivation (22).

The relationship between AR N-C interactions and AR transactivation was equivocal; and whether the influence of AR N-C interaction can totally translate into AR transactivation and/or *in vivo* cell growth remains unclear. Here we provide the evidence to dissect the possible linkages among AR N-C interactions, AR transactivation, AR coregulator function, and prostate cancer cell growth.

RESULTS

AR Coregulators Had Different Effects on AR N-C Interactions, which May Not Always Correlate with Their Coregulator Function

Using a yeast two-hybrid technique with AR N terminus or AR C terminus as bait, we were able to isolate several AR coregulators, such as ARA70 (full length) and ARA70N (amino acids 1~401) (23), gelsolin (29), ARA54 (25), ARA67 (30), and Rad9 (27). Some of these AR coregulators contain a FXXLF motif, which is important for AR N-C and AR-coregulator interactions. We tested all these coregulators plus SRC-1 (31) on AR N-C interactions in a mammalian two-hybrid assay system. As shown in Fig. 1A, ARA70 and ARA54 have no effect, Rad9 can block, and SRC-1 can enhance AR N-C interactions in both COS-1 and PC-3 cell lines. Surprisingly, ARA70N can strongly block and ARA67 can enhance AR N-C interactions in both COS-1 and PC-3 cell lines, and gelsolin can suppress AR N-C interactions in the COS-1 cell line but has no effect in the PC-3 cell. ARA70N can suppress AR N-C interaction in titration dose study. We further tested whether the effects of these AR coregulators on AR N-C interactions can translate their influences to AR transactivation. As shown in Fig. 2A, AR coactivators ARA70, ARA70N, gelsolin, ARA54, and SRC-1 can enhance AR transactivation, and AR corepressors ARA67 and Rad9 can suppress AR transactivation in both COS-1

and PC-3 cell lines. In both Rad9 and SRC-1, effects on AR N-C interactions correlate with their coregulator functions. However, both coactivators ARA70 and ARA54, which contain a FXXLF motif that may play important roles for coregulator-AR interactions, show little effect on AR N-C interactions. Furthermore, AR coactivator ARA70N and gelsolin can suppress, and corepressor ARA67 can enhance, AR N-C interactions, which do not correlate with their coregulator functions. Most of these tendencies can also be seen in LNCaP cell, with either mouse mammary tumor virus (MMTV)-luciferase (LUC) or prostate-specific antigen (PSA)-LUC reporter gene assays except for ARA54 (Fig. 2B). We checked the tested AR coregulator expression level in COS-1 and PC-3 cells and showed all of them can be expressed in these two cell lines at different amounts (Fig. 1B). Together, these data suggested that under the same cell condition, such as in COS-1 and PC-3 cells, AR coregulators can have different effects on AR N-C interactions, which may not always correlate with their coregulator functions.

With Combinations of Some AR Coregulators, AR Transactivation Was Further Enhanced in Contrast to the Effects on AR N-C Interaction

The function of AR can be modulated by different coregulators through various mechanisms. We had tested the effects of individual coregulator on AR N-C interactions, as shown in Figs. 1 and 2, and Table 1. Of primary interest was the type of relationship between AR N-C interactions and AR transactivation with combinations of various coregulators. We tested ARA70N, which functions as a strong AR coregulator, in combination with other AR coregulators. As shown in Fig. 3A and Table 1, the combination of two AR N-C blockers (ARA70N and gelsolin), or one blocker and ARA54, which showed little effect on AR N-C interaction, could further enhance AR transactivation. Both AR corepressors (ARA67 and Rad9) can suppress ARA70N coregulator function and AR coactivator SRC-1 can further enhance AR transactivation. Similar tendencies can also be seen in LNCaP with either MMTV-LUC or PSA-LUC reporter assay (Fig. 3B). These results suggested that the effect of AR coregulator on AR N-C interactions in some conditions may conflict with their effects on AR transactivation.

AR C Terminus-Associated Peptides Can Suppress AR N-C Interaction, which Does Not Correlate with the Degree of Suppression on AR Transactivation

Using phage display, we were able to screen out several AR C terminus-associated peptides containing FXXLF or FXXLF-like motifs (22). Because the FXXLF motif of the AR N terminus is important for the AR N-C

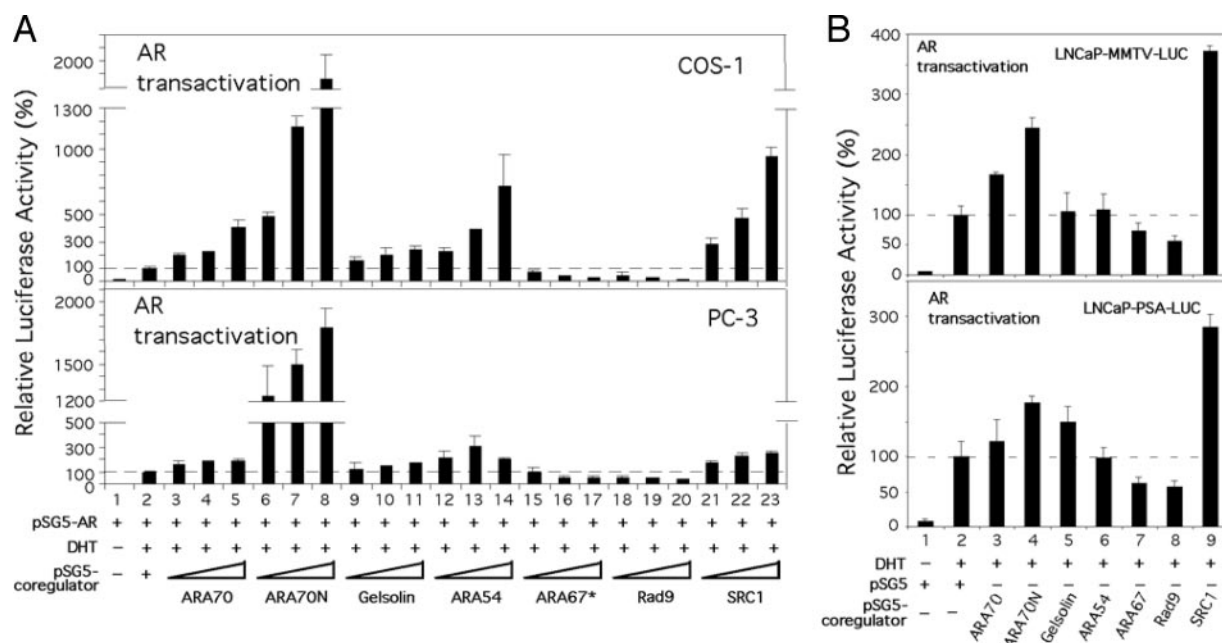


Fig. 2. The Effects of AR Coregulators on AR N-C Interaction May Not Always Correlate with AR Coregulator Function. The same AR coregulators in Fig. 1 were tested for effects on AR transactivation. A, Seventy nanograms of pSG5-hAR, 250, 500, or 700 ng pSG5-AR coregulators, 250 ng MMTV-LUC, and 0.5 ng SV40-Renilla LUC, and additional pSG5 to total 1020.5 ng plasmid in individual wells were transfected into COS-1 and PC-3 cell lines in 24-well plates. B, Seven hundred nanograms of pSG5 or pSG5-AR coregulators, 250 ng MMTV-LUC or PSA-LUC, and 0.5 ng SV40-Renilla LUC were transfected into LNCaP cell line in 24-well plates. After 2 h transfection, the medium was replaced. After 16 h, ethanol or 10 nM DHT was added for another 16 h. A dual LUC assay was performed. Results are mean ± SD of at least three independent assays.

interactions, we were interested to examine whether these motif-containing peptides could block AR N-C interactions. As shown in Fig. 4 and Table 2, the tested peptides can block AR N-C interaction very efficiently in COS-1 and PC-3 cell lines in a modified AR N-C interaction assay. Because AR N-C interactions may play important roles in the full function of AR, we further tested whether these AR N-C interaction-blocking peptides can influence AR transactivation. As shown in Fig. 5 and Table 2, most of the tested peptides can suppress AR transactivation in most tested cell lines but are not so effective compared with their effects on AR N-C interaction.

Selective AR C Terminus-Associated Peptides, which Contain FXXLF or FXXLF-Like Motifs, Can Influence ARA70N Coregulator Function

Both the AR N terminus and some AR coregulators use the FXXLF motif to interact with the AR C terminus AF-2. We tested the effect of some AR C terminus-associated peptides containing FXXLF or FXXLF-like motifs on ARA70N coregulator function. As shown in Fig. 6, most of the tested peptides can partially suppress ARA70N coregulator function, but to different degrees in tested cell lines. Although tested peptides and ARA70N can individually suppress AR N-C inter-

Table 1. Effects of Individual Coregulator on AR-NC Interactions, AR/AR-ARA70N Transactivation

Coregulator, Cell Line	AR N-C Interaction		AR Transactivation				AR-ARA70N Transactivation		
	COS-1	PC-3	COS-1	PC-3	LNCaP		COS-1	PC-3	LNCaP
					MMTV-LUC	PSA-LUC			
1. ARA70FL	→	→	↑	↑	→	→			
2. ARA70N	↓	↓	↑	↑	↑	↑			
3. Gelsolin	↓	→	↑	→	→	→	↑		→
4. ARA54	→	→	↑	→	→	→	↑		
5. ARA67	↑	↑	↓	↓	↓	↓	↓		↓
6. RAD9	↓	↓	↓	↓	↓	↓	↓		↓
7. SRC1	↑	↑	↑	↑	↑	↑	↑		↑

↑, Enhance; ↓, suppress; →, little or no effect.

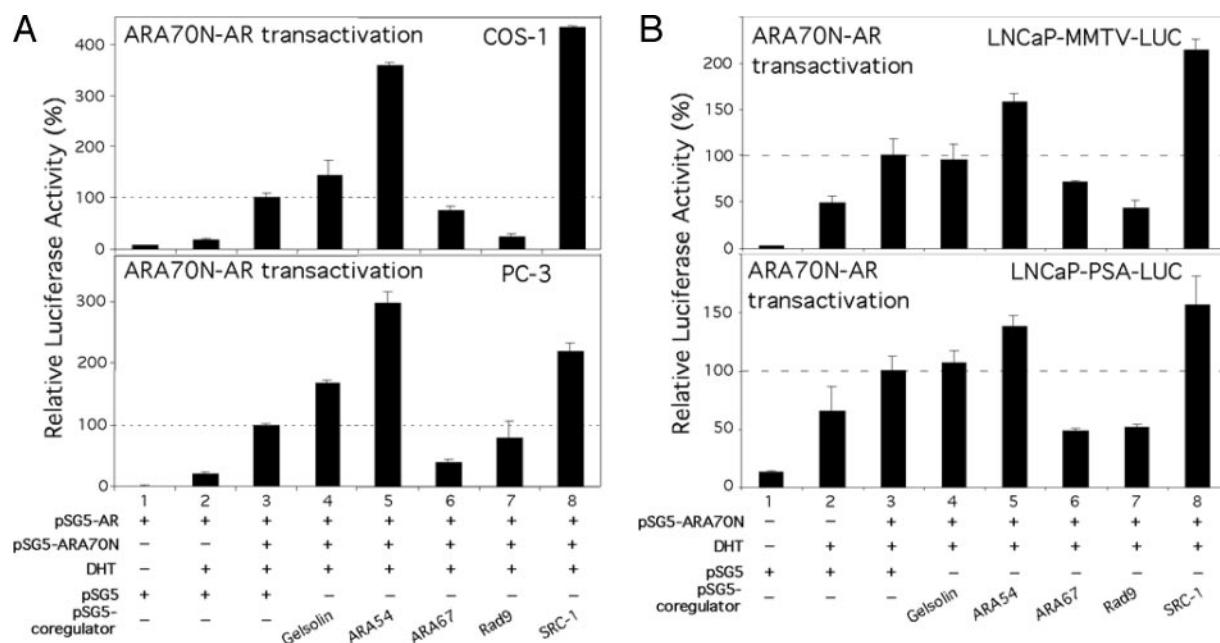


Fig. 3. With Combinations of Some AR Coregulators, AR Transactivation Was Further Enhanced Compared with Their Effects on AR N-C Interaction

The AR coregulators from Fig. 1 were tested for coregulator function in combination with ARA70N. A, In COS-1 and PC-3 cell lines, 30 ng pSG5-hAR, 70 ng pSG5 or pSG5-ARA70N, 250 ng MMTV-LUC, and 0.5 ng SV40-*Renilla* LUC were transfected in 24-well plates, with addition of 700 ng pSG5 or pSG5-AR coregulators to individual wells. B, in LNCaP cell line, 350 ng pSG5 or pSG5-ARA70N, 700 ng pSG5 or pSG5-AR coregulators, 250 ng MMTV-LUC or PSA-LUC, and 0.5 ng SV40-*Renilla* LUC were transfected into 24-well plates. After 2 h transfection, the medium was replaced. After 16 h, ethanol or 10 nM DHT was added for another 16 h. A dual-LUC assay was used. Results are mean \pm SD of at least three independent assays.

actions (Figs. 1 and 4), in combination they can enhance AR transactivation. Together, their effects on AR N-C interactions do not directly translate to effects on transactivation.

AR N-C Interaction Blocking Peptides Have Different Effects on Prostate Cancer Cell Growth

From Figs. 4–6, we found AR C terminus-associated peptides could suppress AR N-C interaction very efficiently but had different effects on AR transactivation. It was interesting to know whether the effects of these peptides on AR transactivation could correlate with their effects on AR-mediated cell growth. We chose four peptides with different effects on AR transactivation, two FXXLF-containing peptides, nos. 3–18 and B310, and two FXXMF-containing peptides, C421 and C312. We synthesized these peptides by conjugating a fragment of transactivating regulatory protein of human immunodeficiency virus type 1 (TAT), which aids the peptide in entering the cytosol, to test their effects on the prostate cancer LNCaP and CWR22R cell lines, both containing endogenous AR. The conjugated peptides achieved good penetration into the tested cells as shown in Fig. 7B. As shown in Fig. 7A, all the tested peptides including TAT alone could suppress LNCaP cell growth but the FXXLF-containing peptides, nos. 3–18 and B310 had better suppression effect. Surprisingly, only those two FXXLF-containing

peptides can suppress CWR22R cell growth in the presence of DHT. Together, these data suggest the suppression of AR N-C interactions may not always correlate with the suppression of AR transactivation or the inhibition of AR-mediated cell growth.

DISCUSSION

AR N-C Interaction Is Unique and Important for Full Function of AR

AR N-C interactions are unique in nuclear receptors and may play important roles in influencing the full function of AR via the following mechanisms: receptor dimerization, stabilization of a ligand in the LBD ligand binding pocket, and retardation of AR degradation (13, 32). Compared with other steroid hormone receptors such as the estrogen receptor (33, 34), AR has a longer N terminus with stronger activation function-1 and weaker AF-2 in the C terminus (12). Although the AF-2 in liganded AR can recruit LXXLL motif-containing coregulators to help transactivation (20), it can also bind with the AR N-terminal FXXLF motif to allow the N terminus to recruit other coregulators to the liganded AR complex. SRC-1 has been found to use its LXXLL motif to interact with the liganded AR C terminus, but its glutamine-rich area to interact with the AR N terminus. Interestingly, the AR N terminus-SRC-1 inter-

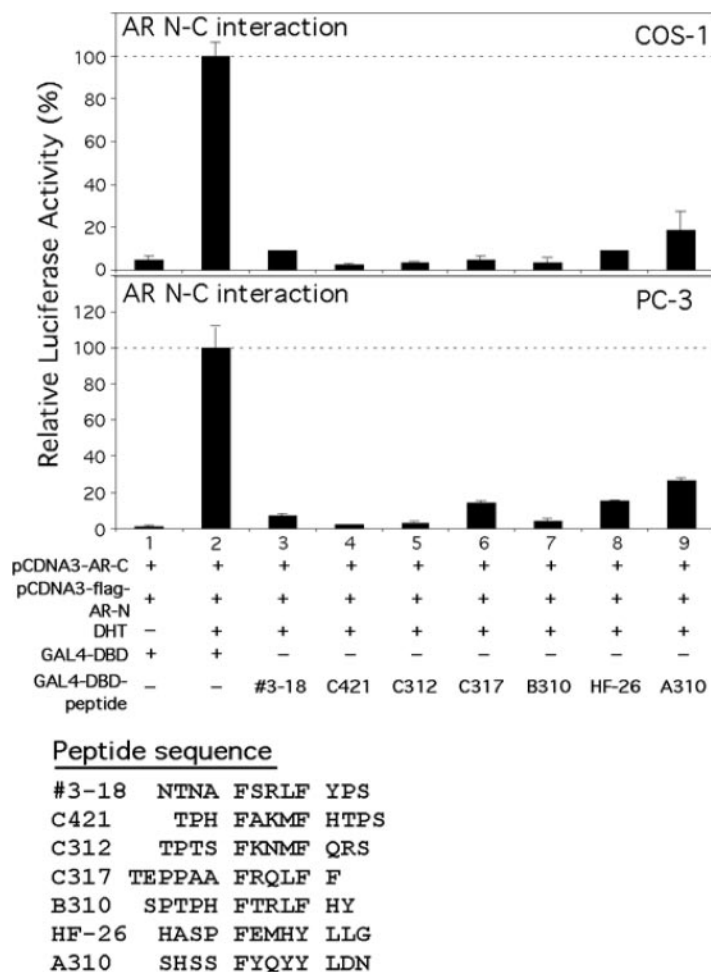


Fig. 4. AR C Terminus-Associated Peptides Can Suppress AR N-C Interactions

Three FXXLF motif-containing peptides, two FXXMF-motif containing peptides, and two FXX(H/Y)Y motif-containing peptides (where F is phenylalanine, H is histidine, M is methionine, X is any amino acid, and Y is tyrosine), from liganded AR C terminus-associated peptides were tested for effects on AR N-C interaction in a modified AR N-C interaction assay (39). Seventy nanograms of pCDNA3-flag-hAR-N (amino acids 1–506) combined with 70 ng pCDNA3-hAR-C (amino acids 556–919), 250 ng MMTV-LUC, 0.5 ng SV40-*Renilla* LUC, and 700 ng GAL4-DBD-peptide plasmids were transfected into COS-1 or PC-3 cell lines in 24-well plates, with GAL4-DBD added to individual wells to make equal amounts of plasmid in every well. After 2 h transfection, the medium was replaced. After 16 h, ethanol or 10 nM DHT was added for another 16 h. Results are mean ± SD of at least three independent assays.

Table 2. Tested Peptides Can Block AR N-C Interaction, AR/AR-ARA70N Transactivation, and Cell Growth

Peptide, Cell Line	AR N-C Interaction		AR Transactivation		AR-ARA70N Transactivation		Cell Growth	
	COS-1	PC-3	COS-1	PC-3	COS-1	PC-3	LNCaP	CWR22R
1. Nos. 3–18	↓	↓	↓	↓	↓	↓	↓	↓
2. C421	↓	↓	↓	↓	↓	↓	↓	→
3. C312	↓	↓	↓	↓	↓	↓	↓	→
4. B310	↓	↓	↓	↓	↓	↓	↓	↓
5. C317	↓	↓	↓	↓	↓	↓	↓	↓
6. HF-26	↓	↓	↓	↓	↓	↓	↓	↓
7. A310	↓	↓	↓	↓	↓	↓	↓	↓

↓, Suppress; →, little or no effect.

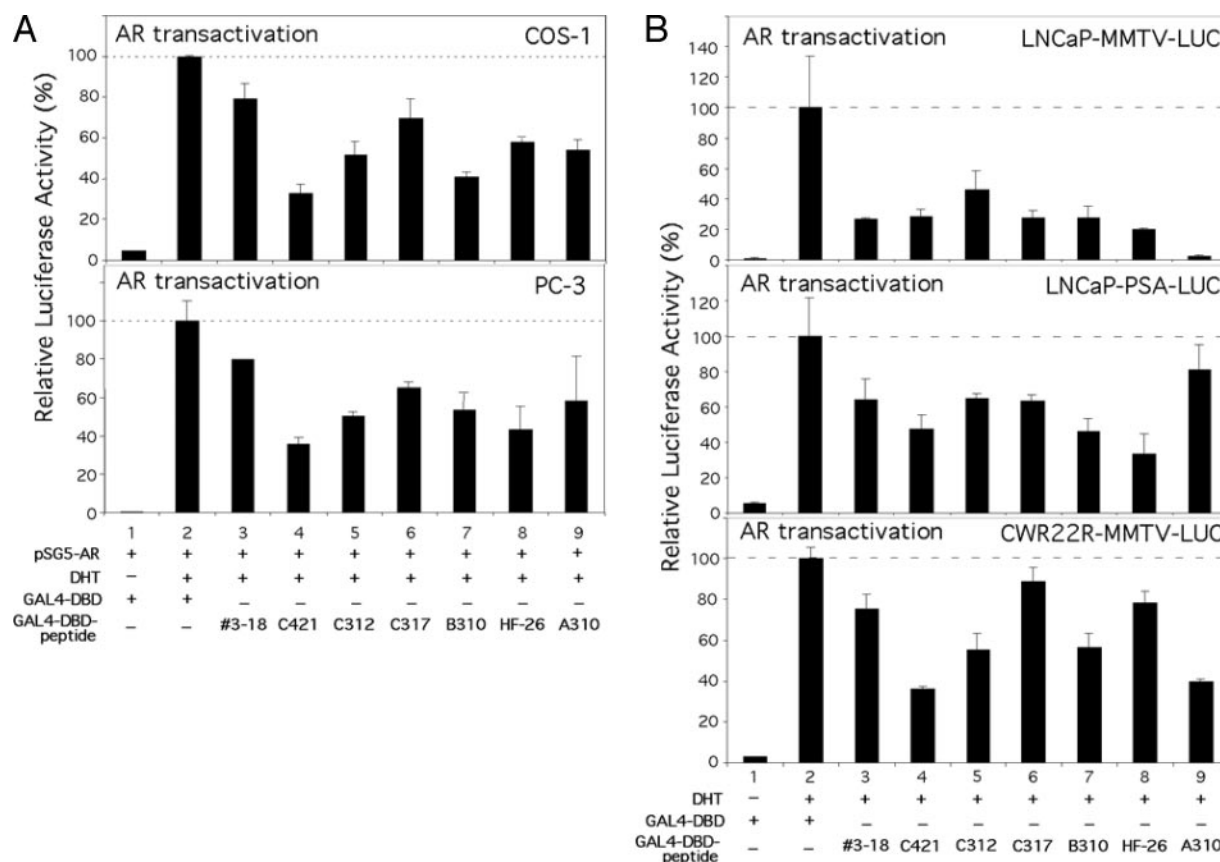


Fig. 5. The Effects of AR C Terminus-Associated Peptides on Suppression of AR N-C Interaction Did Not Always Correlate with the Degree of AR Transactivation Suppression

The same peptides from Fig. 4 were tested for effects on AR transactivation. A, Seventy nanograms of pSG5-hAR, 250 ng MMTV-LUC, and 0.5 ng SV40-*Renilla* LUC were transfected into COS-1 and PC-3 cell lines in 24-well plates, with the addition of 700 ng GAL4-DBD or GAL4-DBD-peptides to individual wells. B, Two hundred fifty nanograms of MMTV-LUC or PSA-LUC, 0.5 ng SV40-*Renilla* LUC, and 700 ng GAL4-DBD or GAL4-DBD-peptides were transfected into LNCaP and CWR22R cell lines in 24-well plates. After 2 h transfection, the medium was replaced. After 16 h, ethanol or 10 nM DHT was added for another 16 h. A dual-LUC assay was used. Results are mean \pm SD of at least three independent assays.

action, but not the LXXLL motif-AR C terminus interaction, was important for AR transactivation (35). As shown in Fig. 1, the microenvironments in the individual cell line may influence AR N-C interactions. These results suggest that the effects of AR-associated proteins/peptides on AR N-C interactions may not always correlate with their effects on AR transactivation, coregulators' function, or AR-mediated cell growth.

AR-Associated Proteins/Peptides Influence AR Transactivation by Multiple Mechanisms

Some reasonable explanations as to why AR N-C interaction effects may not always translate into similar effects on AR transactivation could include the following. First, coactivator ARA70N not only has a strong functional domain that can enhance AR transactivation (23, 36) but also contains one FXXLF motif (amino acids 323–327), which can compete with the AR-N-terminal FXXLF motif to bind the AR-C-terminal AF-2 domain (22, 37, 38). This competition may result in the

blockage of AR N-C interactions. Second, gelsolin can interact with the AR-DBD-LBD via its C terminus in a ligand-dependent manner but cannot interact with AR N terminus that may block AR N-C interaction. Gelsolin, like another actin-binding protein, filamin, may facilitate AR nuclear translocation and enhance AR transactivation (29). Third, ARA67 can interact with both N- and C-termini of the AR, which may then be able to hold AR N-C together to enhance AR N-C interaction. On the other hand, ARA67 may also be able to interrupt AR cytoplasmic-nuclear shuttling, which can result in the suppression of AR transactivation (30). Fourth, the AR C terminus may have weaker binding affinity with its associated peptides than the AR dimer with two intact AR molecules. Although these peptides can block AR N-C interactions, they may not block AR transactivation (22, 39). Fifth, although bound to the AR C terminus, some associated peptides may block N-C interactions; however, the bound complex may then convert the whole AR mol-

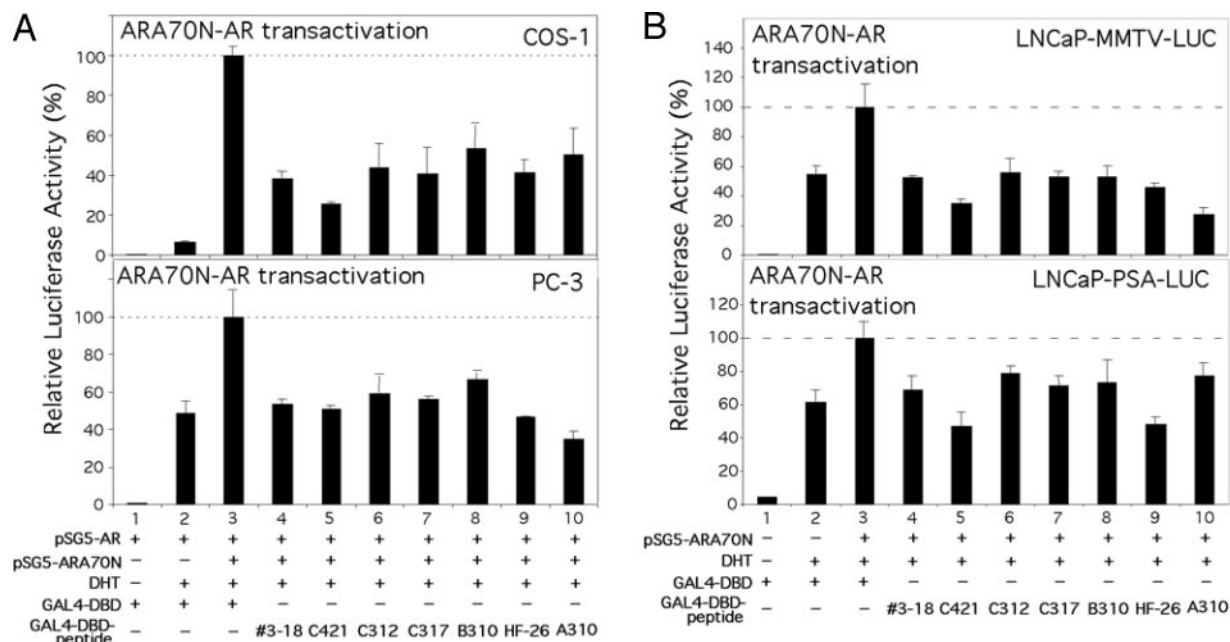


Fig. 6. AR C Terminus-Associated Peptides, which Contain FXXLF or FXXLF-like Motifs, Can Influence ARA70N Coregulator Function

The same AR C terminus-associated peptides from Fig. 4 were tested for effects on ARA70 coregulator function. A, In COS-1 and PC-3 cell lines, 30 ng pSG5-hAR, 70 ng pSG5-ARA70N, 250 ng MMTV-LUC, and 0.5 ng SV40-*Renilla* LUC were transfected in 24-well plates, with addition of 700 ng GAL4-DBD or GAL4-DBD-peptides to individual wells. B, In LNCaP cell line, 350 ng pSG5 or pSG5-ARA70N, 700 ng GAL4-DBD or GAL4-DBD-peptides, 250 ng MMTV-LUC or PSA-LUC, and 0.5 ng SV40-*Renilla* LUC were transfected in 24-well plates. After 2 h transfection, the medium was replaced. After 16 h, ethanol or 10 nM DHT was added for another 16 h. A dual-LUC assay was used. Results are mean \pm SD of at least three independent assays.

ecule to a different active form to recruit more coregulators to enhance AR transactivation (39).

The Effect of AR-Associated Proteins/Peptides on AR N-C Interaction May Not Always Correlate with Their Effects on AR-Mediated Cell Growth

Furthermore, influencing AR N-C interactions may also not directly translate into AR-mediated cell growth due to the following conditions. First, the influence of AR N-C interactions may not always correspond to similar effects with potential reasons mentioned above. Second, AR can go through protein-protein interactions to inhibit the function of some transcription factors, such as nuclear factor κ B, Ets-1, and cAMP-responsive element binding protein (40–42), which may influence cell growth. Third, in LNCaP cells, low concentrations of DHT can stimulate cell growth, whereas high concentrations of DHT can enhance PSA expression (43). Fourth, in different culture conditions, AR may turn on the expression of different target genes (44). Fifth, the microenvironments in the individual cell may influence different AR roles in each cell. For example, in LNCaP cells, enhancing AR transactivation through the addition of androgens can enhance cell growth (45), however, in a bone metastasis, prostate cell line PC-3 stably transfected with AR, such as the PC-AR2 cell line, employing the same method of enhancing AR

transactivation will induce apoptosis (46, 47). Furthermore, in another prostate cancer CWR22R line that contains endogenous AR, addition of androgen may produce only marginal cell growth enhancement (48, 49). The AR in CWR22R cells was found to have a duplication of exon 3, which encodes the second zinc finger of the DBD, which responded very well to androgen in AR target gene reporter assays, but could not express PSA (49). In androgen-refractory prostate cancer cells with endogenous AR, disruption of AR function may inhibit cell growth (50). Recently, one report used the antiprogestin RU486, which produces a weak AR agonist effect, as a prototype drug to test some side chain modifications. They found that some RU486 derivatives can block AR N-C interactions, and enhance or have no effect on AR transactivation but can stimulate prostate cancer LNCaP cell proliferation (51). These results strongly indicate that AR N-C interactions may not always correspond with AR-mediated transactivation and cell growth. Moreover, using co-crystallization of AR DBD with AR response elements, Shaffer *et al.* (52) found that two AR DBD monomers form a dimer in a head-to-head pattern on AR response elements, which contradicts the antiparallel arrangement of AR monomers in AR N-C interactions (53). These controversial data further raise questions about the roles of antiparallel AR N-C interaction in the AR *in vivo* roles. The reason why the tested FXXLF-

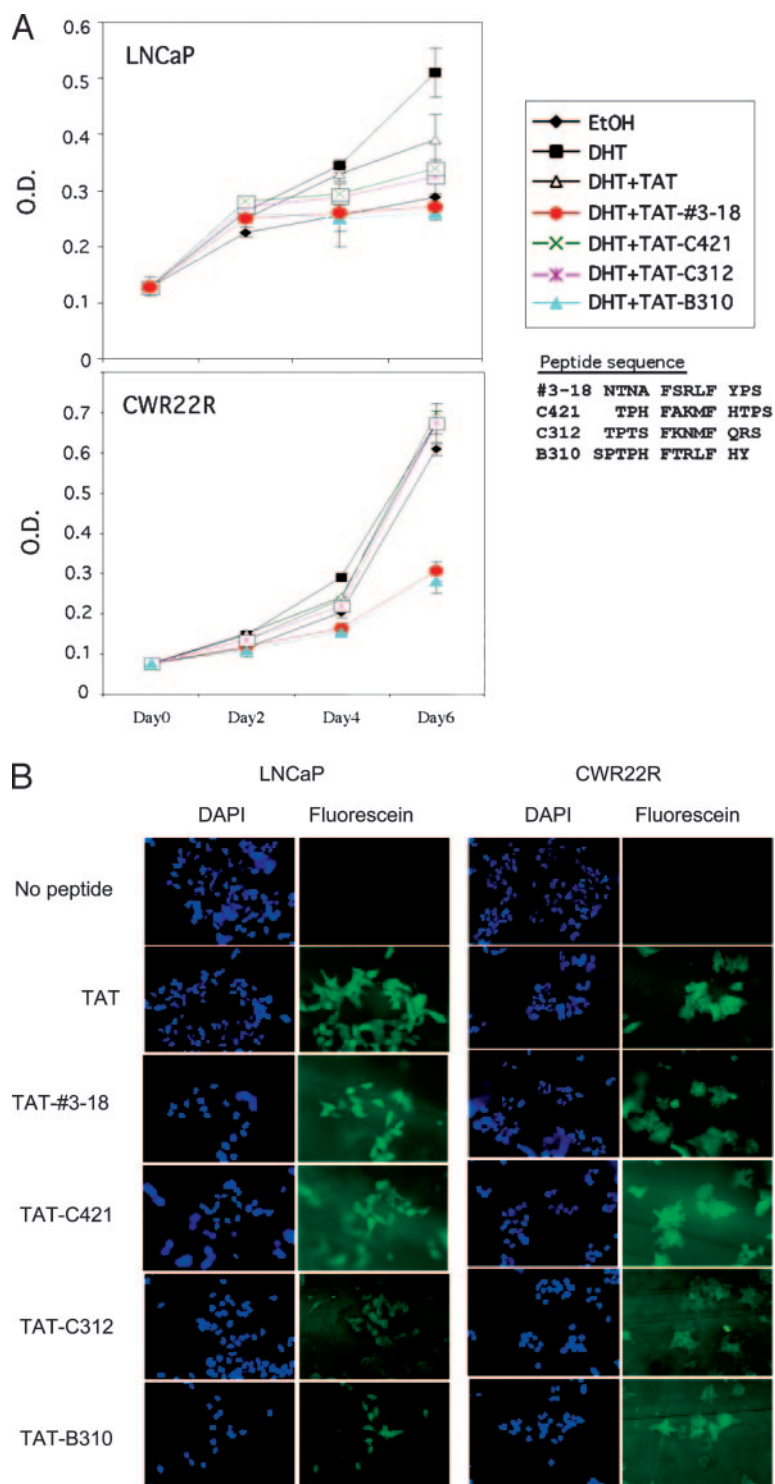


Fig. 7. FXXLF or FXXLF-Like Motif-Containing Peptides Had Different Effects on Prostate Cancer Cell Growth

A, Four peptides, nos. 3–18 and B310 with the FXXLF motif and C421 and C312 with the FXXMF motif, conjugated with TAT, were synthesized. Prostate cancer LNCaP or CWR22R at 5×10^4 cells were seeded in 24-well plates and treated with ethanol, 1 nM DHT, 1 nM DHT with 20 μ M TAT, or 1 nM DHT with 20 μ M TAT peptide on d 2, 4, and 6. At each time point, 50 μ l of 1 mg/ml MTT solution was added to each well containing 500 μ l medium with 3 h incubation and 500 μ l of isopropyl alcohol was added to dissolve the converted dye. The absorbance of each well was measured at 570 and 650 nm using a DU 640B spectrophotometer (Beckman) according to the manufacturer's protocol. B, LNCaP and CWR22R cells were seeded on chamber slides for 24 h. Cells were treated with 1 μ M TAT peptides for 16 h. After washing with PBS, cells were fixed and mounted. The uptake of peptides in cells was detected under fluorescence microscope. Fluorescein signal indicates peptides uptake and 4,6-diamidino-2-phenylindole (DAPI) signal indicates cell nucleus.

containing peptides had better suppression effect on LNCaP and CWR22R cells growth than FXXMF-containing peptides were unknown. Nevertheless, these suppression effects all indicate AR N-C interaction did not correlate well with AR function.

It is therefore unpredictable to assume *in vivo*/physiological AR function solely based on the assay of AR N-C interactions, even though these interactions may play some roles for full AR function *in vitro*. Drug designs based on the modulation of AR N-C interactions may not have equivalent influence on *in vivo* AR function. To successfully screen drugs to battle androgen/AR-related diseases such as prostate cancer, further examinations of AR physiological functions through *in vivo* interventions are necessary.

Materials and Methods

Materials and Plasmids

DHT and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO). Green monkey kidney fibroblast COS-1 and human prostate cancer cell lines, PC-3 and LNCaP, were purchased from ATCC. Prostate cancer CWR22R cell line was a gift from Dr. Franky Chan of Hong Kong University. Peptides were synthesized by Alpha Diagnostics International and purified by HPLC to greater than 95% purity and confirmed by mass spectrometry analysis. The peptide sequences are as follows: fragment of TAT, fluorescein-GGGYGRKKRRRQRG; TAT nos. 3–18, fluorescein-GGGYGRKKRRRQRG-NTNA-FSRLF-YPS; TAT-C421, fluorescein-GGGYGRKKRRRQRG-TPH-FAKMF-HTPS; TAT-C312, fluorescein-GGGYGRKKRRRQRG-TPTS-FKNMF-QRS; TAT-B310, fluorescein-GGGYGRKKRRRQRG-SPTPH-FTRLF-HY. All peptides were dissolved in water. pSG5-AR, pSG5-ARA70N (ARA70 N terminus, amino acids 1–401), pSG5-ARA70 (23), pSG5-gelsolin (29), pSG5-ARA54 (25), pCDNA3-RAD9 (27), pSG5-ARA67, pSG5-SRC1, pCMX-GAL4-AR-C, pGL3-PSA6.0-LUC (PSA-LUC) (30), and pCMX-GAL4-peptides (22) were constructed as described in our previous publication. PCR was used to construct AR-N (amino acids 1–506) via *Bam*HI/*Kpn*I to pCDNA3-flag and pCMX-VP16 vectors and AR-C (amino acids 556–919) via *Bam*HI/*Xba*I into pCDNA3.

Mammalian Two-Hybrid Assay

For the LUC assay, 250 ng pG5-LUC reporter gene plasmid, 0.5 ng simian virus 40 (SV40)-*Renilla* LUC internal control plasmid, 700 ng coregulator in pSG5 or pCDNA3 vector, 70 ng pCMX-GAL4-AR-C, and 70 ng VP16-AR-N were transfected into COS-1 and PC-3 cells with Superfect kit (QIAGEN, Valencia, CA). After 2 h transfection, the medium was replaced. After 16 h, ethanol or 10 nM DHT were added to the wells for another 16 h. The dual LUC reporter assay system (Turner Designs, Sunnyvale, CA) was employed to measure LUC activity.

Transfection and Reporter Gene Assay

COS-1 and PC-3 cell line were grown in DMEM containing 10% fetal calf serum and LNCaP and CWR22R cell lines were maintained in RPMI-1640 (Invitrogen Life Technologies, Carlsbad, CA) with 10% fetal calf serum. For transfection, the cells were plated in 24-well dishes and plasmids with 20–70

ng pSG5-AR, 0–70 ng pSG5-ARA70N, 700 ng pSG5-coregulator, 250 ng MMTV-LUC or PSA-LUC, and 0.5 ng SV40-*Renilla* LUC internal control plasmid were transfected by Superfect kit as described previously. pSG5 vector was added to make plasmid amounts equal for each transfection. After 2 h transfection, the medium was replaced. After 16 h incubation, the cells were treated with ethanol or 10 nM DHT for another 16 h and then harvested for the dual LUC assay. The MMTV-LUC reporter gene and PSA-LUC was used to measure AR transcriptional activity, and a SV40-*Renilla* LUC plasmid (Promega, Madison, WI) was used as an internal control. The dual-LUC reporter assay system (Turner Designs) was employed to measure the LUC activity.

Cell Growth Assay

Cell growth was measured by MTT assay (54). Cells were plated at 5×10^4 cells/well in 24-well plates and incubated with or without 1 nM DHT and 20 μ M peptide for 2–6 d. At each time period, 50 μ l of 1 mg/ml MTT solution was added to each well containing 500 μ l medium with 3 h incubation and 500 μ l isopropyl alcohol was added to dissolve the converted dye. The absorbance of each well was measured at 570 and 650 nm by using a DU 640B spectrophotometer (Beckman, Fullerton, CA) according to the manufacturer's protocol. Values in the figure are the means \pm SD of OD₅₇₀-OD₆₅₀ from at least three independent wells of cells.

Fluorescence Microscopy

LNCaP and CWR22R cells were seeded on two-well Lab Tek Chamber slides (Nalge Nunc International, Rochester, NY) in RPMI-1640 with 10% CD-FBS for 18 h before being treated with 1 μ M TAT peptides. After treatment with TAT peptides for 16 h, cells were fixed in fixation solution (3% formaldehyde and 10% sucrose in PBS) and were then washed, mounted with VECTASHIELD mounting medium with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), and photographed under 400-fold magnification with a fluorescence microscope.

Western Blot Analysis

COS-1 and PC-3 cells were seeded on a 100-mm dish 1 d before transfection. Cells were transfected with 10 μ g pSG5-coregulators by Superfect or 30 μ g pSG5-ARA67 by calcium phosphate precipitation method. After 24 h, total cell lysates were prepared by lysing cells in ice-cold RIPA buffer (1% Igepal CA-630, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate in PBS). The lysates were clarified by centrifugation. The protein concentration of the supernatant was evaluated with the Bio-Rad Laboratories (Hercules, CA) reagent kit. From each sample, 100 μ g proteins were separated by 6 or 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in TBST [10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.05% Tween 20] containing 5% nonfat dry milk for 1 h at room temperature. Primary antibodies, anti-ARA70 (CC70 3), anti-ARA54 (N-17) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-gelsolin (GS-2C4) (Sigma), anti-ARA67 (mAb26/37) (55), anti-RAD9 (M-389) (Santa Cruz), anti-SRC-1 (C-20) (Santa Cruz), and antiactin (I-19) (Santa Cruz), were added and incubated at 4 C overnight. The alkaline phosphatase-conjugated secondary antibodies (Santa Cruz) in TBST were added and incubated for 1 h at room temperature. The membranes were washed three times in TBST, and the immunoreactive bands were visualized by alkaline phosphatase activity with the 5-bromo-4-chloro-3-indolylphosphate-nitro blue tetrazolium phosphatase substrate (Bio-Rad Laboratories).

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