Novel Function of Androgen Receptor-associated Protein 55/Hic-5 as a Negative Regulator of Smad3 Signaling

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Androgen receptor-associated protein 55 (ARA55/Hic-5) belongs to the LIM protein superfamily and is featured by three or four N-terminal LD motifs and four C-terminal zinc finger-like LIM domains. Both LD motifs and LIM domains can serve as protein-protein interaction interfaces. Recently, we found that enforced expression of ARA55 inhibits transforming growth factor-β-mediated up-regulation of Smad binding element-luciferase reporter activity in NRP-154 and NRP-152 rat prostate and LNCaP human prostate cell lines. Moreover, ARA55 also inhibits the induction of Smad-binding element 4-luciferase and 3TP-luciferase (a plasminogen activator inhibitor-1 (PAI-1) promoter construct) reporter activity by constitutively active (CA)-Smad3 in these cell lines. Co-immunoprecipitation studies suggest an interaction between ARA55 and either CA-Smad3 or wild-type Smad3 in HEK293 cells that occurs through the MH2 domain of Smad3 and the C terminus of ARA55 with wild-type Smad3 having stronger affinity than CA-Smad3 to ARA55. Glutathione S-transferase pull-down assays demonstrate that this interaction can occur in a cell-free system. These results are consistent with the luciferase data showing that the C terminus of ARA55 is critical for suppression of Smad3 activity. Furthermore, using a mammalian two-hybrid system, we confirmed that ARA55 interacts with the MH2 domain of Smad3 and suppresses CA-Smad3-induced transcriptional responses. In conclusion, these results support that ARA55 selectively intercepts transforming growth factor-β signaling through an interaction of the LIM domain of ARA55 with the MH2 domain of Smad3.

Transforming growth factor-β/Smad signaling (TGF-β/Smad) for which there are three mammalian isoforms are 25-kDa dimeric peptides involved in multiple biological processes such as cell proliferation, cell cycle arrest, differentiation, apoptosis, angiogenesis, wound healing, and immunosuppression (1–3). Two transmembrane serine/threonine kinase receptors, TβRI and TβRII, mediate TGF-β signaling (4–8). The binding of TGF-β1 to TβRII promotes recruitment of TβRI to TβRII to form a heteromeric receptor/ligand complex. A constitutively active kinase domain of TβRII then phosphorylates the glycine-serine domain of TβRI, promoting the activation of the latter receptor (5). With the help of the Smad anchor for receptor activation protein, Smad2 and possibly Smad3 are recruited to TGF-β receptor complex (9). The activated TβRI kinase then phosphorylates the two-terminal serines of the receptor-associated Smads (10), thereby promoting their open conformation. In this state, these receptor-activated Smads can homodimerize, associate with the co-Smad, Smad4, and translocate to the nucleus where they bind to SBE (11–14). Another Smad that associates with TβRI is Smad7; however, this homologous protein instead functions as a negative regulator of TGF-β signaling by recruiting the E3 ubiquitin ligase SmurF1 to promote proteasomal degradation of TβRI (15, 16).

In the mature prostate, the expression and activation of TGF-β and Smads 2 and 3 are suppressed by physiological levels of androgens (17–19). Following castration-induced androgen withdrawal, a robust induction in the expression of TGF-βs and activation of Smads 2 and 3 occur in the prostate, concomitant with the onset of apoptosis and involution (17–21). Both in vivo and in vitro studies show that TGF-β is a potent inducer of the apoptosis, growth arrest, and differentiation of prostate epithelia (22–27) and thus implicate a critical role of TGF-β signaling on the dependence of androgen for cell survival. Upon malignant transformation, prostate epithelial cells acquire resistance to TGF-β-induced apoptosis and growth arrest, events correlating with the down-regulation of TGF-β receptor levels and loss of androgen dependence (28–30). The loss of the ability of TGF-β to promote growth arrest or and induce apoptosis of prostate epithelium may also occur through the androgen receptor (AR) (31) or insulin-like growth factor-1 (32) signaling pathways activated during carcinogenesis (33–35). A number of recent in vivo studies demonstrate that the loss of TGF-β signaling may play a causal role in carcinogenesis of the prostate (35–39).

Numerous transcription factors and co-regulators have been recently identified to physically associate with Smads (40–44). Included in this list is the AR in which our group and others (31, 45–48) have reported to cross-talk with TGF-β through an interaction of Smad3 with AR. Without trophic support of androgenic steroids, the prostate undergoes severe atrophy (49). The binding of androgen to AR causes this receptor to homodimerize, which promotes its nuclear entry and subsequent transcription activation function. Previous work from our laboratory showed that ligand-bound AR blocks TGF-β signals in...
the prostatic carcinoma cell lines, NRP-154 and LNCaP, through the direct association of Smad3 to the ligand-binding domain of AR (31). However, this interaction appears to be cell line-specific because AR does not block Smad3 signals in the NRP-152 rat non-tumorigenic cell line.2 These data led us to hypothesize that AR co-regulators may play a role in such differential inhibition.

We thereby explored whether the AR-associated protein ARA55, also called Hic-5 (hydrogen peroxide-inducible clone-5), could play such a role by controlling suppression of TGF-β signals by AR. ARA55 was originally identified as a TGF-β or hydrogen peroxide-induced protein in mouse osteoblast MC3T3-E1 cells in which its overexpression promotes cellular senescence (50). As a member of the LIM protein superfamily, ARA55 is featured by four conserved zinc-finger-like LIM domains in its C terminus (Fig. 1A) and homology to the well-defined focal adhesion adaptor/scaffold protein paxillin (51). The N terminus of ARA55 has another conserved region named LD motif (L6XL6XXL). To date, two ARA55 isoforms have been identified that differ by an extra LD motif in the longer isoform (52). Initial studies on ARA55 focused on its negative roles in integrin-mediated signaling. Two focal adhesion-related kinases, FAK (53) and PYK2 (54), have been identified as ARA55 LD motif-binding proteins. ARA55 can compete with paxillin to interact with FAK in NIH 3T3 cells, leading to reduced cell spreading on fibronectin (55). Therefore, ARA55 may moderate proliferation signals transduced from focal adhesions to the nucleus in normal cells. As such, the loss of this LIM domain protein, as seen in carcinomas, may be permissive to certain malignancies (56).

Subsequent efforts revealed that ARA55 can associate with such steroid hormone receptors as AR, glucocorticoid receptor, or progesterone receptor through its C terminus and enhance their transactivation in a steroid hormone-dependent manner (57), probably facilitating cell proliferation. These two discrepant functions of ARA55, promoting either cell senescence or cell growth, may be explained by the finding that the phosphorylation of ARA55 at tyrosine 43 by PYK2 can cause ARA55 to selectively lose its activity for AR transactivation (58) and possibly for transactivation by other steroid hormone receptors (57). Thus, the differential functions of ARA55 may be determined by its phosphorylation state. In this case, PYK2 may be more involved in regulating the activity of ARA55 than that of FAK as recent work suggests that ARA55 is not phosphorylated by FAK (53). In this study, we report that Smad3 is another important target of ARA55 in which this LIM protein effectively inhibits gene transcription by Smad3 or TGF-β. Experimental support is provided such that transcriptional suppression occurs through a direct interaction of the LIM domains of ARA55 with the MH2 domain of Smad3. Although the full physiological implications of this interaction are yet to be revealed, it is likely that ARA55, which is induced by TGF-β in certain cells types, may function in a negative feedback loop to control TGF-β responses.

EXPERIMENTAL PROCEDURES

Plasmids— cDNA fragment of ARA55 (the short isoform) and its truncations were amplified by PCR with ThermalAgaDNA polymerase (Invitrogen) using a human prostatic cDNA library (pLIB-prostate, Clontech) as the template and primers (Integrated DNA Technologies) listed in Table I. The resulting PCR products were inserted into FLAG or Myc (5′) tagged pcDNA3 vector (31). WT-Smad3 and its truncations were also amplified by PCR (High Fidelity PCR Kit™, Roche Applied Science) from pLIB-prostate library with the primers in Table II, and resulting PCR products were inserted into pcDNA3-FLAG vector. CA-Smad3 was prepared by site-directed mutagenesis of serines to aspartic acids in its C-terminal SSXS phosphorylation motif (31). Vectors encoding Gal4 DNA-binding domain (BD), NF-κB transcription activation domain (AD), and the Gal4 luciferase reporter were from a mammalian two-hybrid assay kit (Stratagene). ARA55 or CA-Smad3 was inserted into the BD vector. Smad3 MH1 domain-middle linker (ML) region (MH1-ML) or Smad5 MH2 domain-middle linker (ML) region (MH2-ML) was inserted into the AD vector. All of the constructs were confirmed by DNA sequencing (Cleveland Genomics, Inc.) and by Western blot analysis of transfected HEK293 cells.

Cell Passaging—NRP-154 (passages 18–28) and NRP-152 (passages 55–65) rat prostatic cell lines were cultured in GM2 medium (59) and passed every 3 days at subconfluence. The HEK293 (passages 42–52) cell line was cultured in DMEM/F-12 (Invitrogen) supplemented with 10% FBS (Hyclone). RNA Preparation and RT-PCR—RNA was extracted from NRP-154 and NRP-152 cells cultured overnight in GM3 medium (DMEM/F-12 (v/v), 1% calf serum, 0.1 mM sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing 1% calf serum, 0.1 mM deoxymethasone, 15 mM HEPES, pH 7.4 (24) and purified using RNasey Mini kit (Qiagen). Total RNA (3.0 μg) was reverse-transcribed with Superscript II kit (Invitrogen) using the (dT)18 primer. Two μl of the resulting RT product was used as template to amplify a 1-kb fragment of rat ARA55 with PCR Master Mix™ kit (Promega). The primer pair for PCR reaction was as follows: sense primer, 5′-GAC AGG CTT ATA ACC ACT GTC CCT TCC AGC-3′; antisense primer, 5′-GAG GAT CCC AGC CAA AGA GCT TCA GGA AGC-3′. PCR amplification conditions were set at 95 °C for 15 s, 65 °C for 30 s, and 72 °C for 1 min (40 cycles).

Transfection of NRP-154 and NRP-152 Cells and Luciferase Assay—Unless indicated, cells were plated overnight at a density of 6 × 10⁴ cells/ml/well in 12-well plates in GM2 medium and then transfected with roughly 1 μg of total plasmid containing 0.4 μg/well reporter vector and 12.5 ng/well pCMV-RL internal control vector (Promega) by a calcium phosphate co-precipitate method as detailed previously (31). Afterward, transfection medium was replaced with 1 ml of GM3 and dual luciferase assays (Promega) were performed 24 or 48 h after transfection using a dual beam luminometer as described previously (31). Data were expressed as Renilla luciferase units, which was calculated from the ratio of firefly luciferase to Renilla luciferase units normalized to untreated control.

GST Pull-down Assay—HEK293 cells plated in 5% FBS-DMEM/F-12 medium in 6-well dishes were transfected with pcDNA3-Myc-ARA55 by a standard calcium phosphate co-precipitate method using 2 μg of DNA/well (26, 60). Cells were harvested and lysed in cold RIPA buffer (150 mM NaCl, 20 mM sodium phosphate, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing 1× Complete Mini-EDTA-free protease inhibitor mixture (61), 1 mM phenylmethylsulfonyl fluoride, and 1 μM Na3VO4 (31). After centrifugation (10 min, 14,000 × g), the cleared lysate was mixed with purified GST-

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Smad3 or GST in cold RIPA buffer and incubated overnight at 4 °C with gentle shaking in the presence of glutathione-Sepharose (Amersham Biosciences). The resin was then centrifuged (5 min, 3000 rpm) and washed three times with cold RIPA buffer. The GST-Smad3-ARA55 complex was eluted with 50 μl of 1x SDS/2-mercaptoethanol loading buffer at 70 °C for 10 min. Eluted proteins were resolved with 4–12% NuPAGE Bis-Tris gel (Invitrogen), transferred to nitrocellulose membrane (0.45 micropores, Millipore), and then immunoblotted using the rabbit polyclonal antibody A-14 against Myc (1:1000, catalog number sc-789, Santa Cruz Biotechnology). Co-immunoprecipitation (co-IP) and Western Blot—HEK293 cells were plated overnight in 10% FBS-DMEM/F-12 in 6-well plates at a density of 5 x 10^5 cells/well. Cells were transfected with 2 μg of DNA/well by a calcium phosphate co-precipitate protocol (31), and 48 h later, the tagged proteins were co-immunoprecipitated with M2 anti-FLAG IgG1 monoclonal antibody (Sigma) and detected by Western blot analysis as described previously (31). Primary antibodies for Western blot analysis were anti-Myc (1:1000, catalog number sc-789, anti-FA1-1 (1:200, catalog number sc-8979, Santa Cruz Biotechnology), and anti-FLAG M1 (Sigma), anti-β-actin (1:40,000, Sigma), and anti-cyclin D2 (1:3000, catalog number sc-593, Santa Cruz Biotechnology). All of the secondary antibodies were obtained from Jackson Immunoresearch Laboratories.

RESULTS

The Relative mRNA Level of ARA55 in NRP-154 and NRP-152 Cell Lines—Previously, we found that ligand-bound AR can repress SBE4Δ18-luciferase reporter activity induced by CA-Smad3 (31). This occurred in NRP-154 and LNCaP cells but not in NRP-152 cells. We hypothesized that AR-associated proteins may be involved in reversing the repressive effect of ligand-bound AR on Smad3-mediated gene expression. Recent reports have shown (62–64) that expression of ARA55 is lower in prostate tumors than in normal prostate, implicating that this AR co-activator is a potential regulator of the effects of AR on Smad3. We examined the relative levels of ARA55 mRNA in the tumorigenic NRP-154 and non-tumorigenic NRP-152 rat prostatic epithelia cell lines by RT-PCR. A 1-kb PCR fragment of ARA55 was amplified from both of these cell lines (Fig. 1B). However, this product was substantially stronger in NRP-152 than in NRP-154 cells. Moreover, the basal level of ARA55 may be weakly induced after 8 h of TGF-β treatment in NRP-154 cells but did not appear to be induced by this treatment in NRP-152 cells (data not shown).2

ARA55 Blocks Transcriptional Activation by either CA-Smad3 or TGF-β—To test the possibility that ARA55 controls the ability of AR to suppress Smad3-mediated transcription, NRP-154 cells were transfected with the reporter plasmid SBE4Δ18-luciferase and various combinations of the expression plasmids for CA-Smad3, AR, ARA55, or the corresponding empty plasmids as control, and then cultures were treated with 10 nM DHT or vehicle. On the next day, cells were supplemented with 10 nM DHT or vehicle control and luciferase activity was measured 24 h later. Transfection of CA-Smad3 caused SBE4Δ18-luciferase reporter induction, and as described previously (31), this induction was blocked by ligand-bound AR (Fig. 2A). The expression of ARA55 further reduced CA-Smad3 activity to basal level. Unexpectedly, such inhibition by ARA55 was not lost in the absence of AR or DHT (Fig. 2A, bars 6 and 7), suggesting that such suppression was independent of both DHT and AR.

In contrast to ARA55, the enforced expression of neither paxillin nor zyxin altered the activation of SBE4Δ18-luciferase by CA-Smad3. We further characterized the ability of ARA55 to suppress Smad3-dependent TGF-β responses using a PAI-1 promoter reporter, 3TP-luciferase, which is widely used and highly TGF-β-responsive (65). In both NRP-154 and NRP-152 cells, transfection of CA-Smad3 (Fig. 2C) and TGF-β (data not shown)3 in NRP-152 cells. To examine whether this inhibition is a common feature of LIM proteins, we developed pcDNA3-based Myc-paxillin and Myc-zyxin expression constructs and examined their effects as above (Fig. 2D). In contrast to ARA55, the enforced expression of neither paxillin nor zyxin altered the activation of SBE4Δ18-luciferase by CA-Smad3.

We further explored the effect of overexpressing ARA55 in LNCaP cells on the expression of TGF-β-regulated proteins by Western blot analysis. For this experiment, LNCaP cells were efficiently transfected with pCMV5-TβRII and either

![Fig. 1. ARA55 structure and relative expression levels in NRP-154 and NRP-152 cells.](https://example.com/ara55_structure.png)
ARA55 Blocks Smad3 Activity

Fig. 2. ARA55 blocks SBE4-reporter activity induced by either CA-Smad3 or TGF-β. A, ARA55 blocks Smad3-induced SBE4-luciferase activity in an AR-independent manner. NRP-154 cells were plated in DMEM/F-12 medium supplemented with 5% dextran-coated charcoal-stripped FBS in 12-well plates overnight. Cells were then transiently co-transfected with a total of 1.042 μg of plasmid DNA consisting of 0.2 μg of SBE4-Luc-reporter, 12.5 ng of pCMV-RL (internal control), and various combinations of expression plasmids for pCMV2-CA-Smad3 (30 ng), pCMV5-AR (0.2 μg), pcDNA3-FLAG-ARA55 (0.6 μg), or empty control vectors followed by ±10 nM DHT treatment for 24 h. B, ARA55 blocks activation of SBE4-luciferase by TGF-β1 in NRP-154 cells. Cells (6 × 10⁴) were plated in 12-well dishes with 1.0 ml of GM2, and after overnight attachment, they were co-transfected with a total of 1.0125 μg of plasmid consisting of 0.5 μg of SBE4-luciferase, 12.5 ng of pCMV-RL, and 0.5 μg/well pcDNA3-FLAG-ARA55 (or pcDNA3 control). Following transfection, medium was replaced with 1.0 ml of GM3. Twelve hours later, cells were treated with 10 ng/ml TGF-β1 (recombinant human TGF-β1, R&D Systems, Inc.) or vehicle (4 mM HCl, 1 mg/ml bovine serum albumin), and dual luciferase measurements were assessed 24 h later. C, in NRP-152 cells, ARA55 inhibits CA-Smad3-mediated induction of SBE4-luciferase. Cells plated as in B were transfected with 1.0125 μg of plasmid DNA consisting of 0.4 μg of SBE4-luciferase, 12.5 ng of pCMV-RL ± 0.1 μg of CA-Smad3, ±0.5 μg of FLAG-ARA55, or corresponding empty vectors. D, other LIM proteins, paxillin and zyxin, have no such inhibitory effect on CA-Smad3-induced SBE4-luciferase activity in NRP-154 cells. Cells plated as in B were transfected with 1.0125 μg of plasmid DNA consisting of 0.4 μg of SBE4-luciferase, 12.5 ng of pCMV-RL ± 0.1 μg of CA-Smad3, and 0.5 μg of pcDNA3-Myc-zyxin, or pcDNA3 empty vector. All of the determinations are the average of triplicate determinations ± S.E. The results shown are representative of three independent experiments. R.L.A., relative luciferase activity.

ARA55 Physically Interacts with Smad3—As mentioned above, many proteins have been identified to bind to ARA55 through either N-terminal LD motifs or C-terminal LIM domains (54, 57, 67–73). We speculated that the inhibitory effect of ARA55 on Smad3 results from a direct interaction between ARA55 and Smad3. Using co-IP experiments, we tested the possible binding between ARA55 and Smad3, Smad2, Smad4, or CA-Smad3. HEK293 cells were co-transfected with pcDNA3 expression plasmids for each of the FLAG-tagged Smad proteins together with Myc-tagged ARA55 or Myc-tagged paxillin. Twenty-four hours later, the FLAG-tagged proteins were immunoprecipitated with anti-FLAG IgG and Myc-tagged ARA55 was detected by immunoblotting. This experiment revealed a physical interaction between ARA55 and WT-Smad3, CA-Smad3, or Smad4 (Fig. 5A), although the latter two interactions appeared weaker. Moreover, when FLAG-ARA55 was immunoprecipitated with anti-FLAG IgG, Myc-WT-Smad3 was co-immunoprecipitated (Fig. 5B).

The Binding of ARA55 and Smad3 Can Occur in a Cell-free System—A GST pull-down assay was done to test whether ARA55 can bind to Smad3 in vitro. Lysates prepared from HEK293 cells transfected with either Myc-ARA55 or control

FLAG-ARA55-pcDNA3 or pcDNA3 empty vector alone. On the next day, cells were treated with 10 ng/ml TGF-β1 or vehicle control, and 48 h later, cells were lysed in RIPA buffer. Western blot analysis of total cell lysates from the above revealed that ARA55 selectively blocks the ability of TGF-β to induce the expression of PAI-1 (Fig. 3A), but not the loss of cyclin D2 expression (data not shown).

These results suggest that ARA55 selectively suppresses some TGF-β-induced responses while not affecting others.

ARA55 Can Block Gal4-CA-Smad3 MH2-ML Activity—ARA55 was previously reported to bind to specific DNA sequences (66), suggesting that this protein may bind directly to SBE and block interaction of Smad3 to SBE. To test this possibility, we developed a DNA construct encoding a fusion protein Gal4 BD-CA-Smad3. Transfection of NRP-154 cells with this construct resulted in the induction of the Gal4-luciferase reporter activity. Co-transfection of FLAG-ARA55-pcDNA reduced this induction relative to the empty vector control (Fig. 4), consistent with the results shown above. Thus, Smad3 or another Smad protein, but not SBE, is the target of ARA55-mediated inhibition.
were incubated overnight at 4 °C with either GST or purified GST-Smad3 fusion protein in the presence of glutathione-Sepharose resin. GST-Smad3 was then pulled down by centrifugation, and the material eluted from the washed resin was immunoblotted with antibody against Myc. The above experiment showed that the binding of ARA55 to Smad3 can occur under cell-free conditions (Fig. 5B).

The MH2 Domain of Smad3 Is Responsible for the Interaction of ARA55 with Smad3—

Smad3 has a N-terminal MH1 domain and a C-terminal MH2 domain, separated by a ML region (Fig. 5D) (3). The MH1 domain is responsible for recognizing and binding to SBE (74), whereas the MH2 domain recruits other transcription factors that initiate transcription (75). To define the individual domains of Smad3 binding to ARA55, we developed a series of pcDNA3-based constructs encoding FLAG-tagged Smad3 truncations (Fig. 5D). HEK293 cells were co-transfected with Myc-tagged ARA55 and each of the FLAG-tagged Smad3 truncations, and 24 h after transfection...
tion, cell lysates were subjected to immunoprecipitation analysis as in Fig. 5A. The M2 mouse monoclonal antibody against FLAG captured the complex of ARA55 with either Smad3 MH2 alone or Smad3 MH2-ML, whereas the latter seemed to have a stronger affinity for ARA55 (Fig. 5E). This finding suggests that MH2 domain is required for the ARA55-Smad3 interaction, whereas ML domain may enhance this binding. We next used a mammalian two-hybrid system to test whether the physical interaction between ARA55 and the MH2-ML region of Smad3 occurs in intact cells. In this experiment, NRP-154 cells were co-transfected with the construct encoding BD-ARA55 fusion protein, the construct encoding AD fused to either the MH2-ML or MH1-ML of Smad3, and the Gal4-luciferase reporter. Co-transfection of the BD-ARA55 construct with AD-MH2-ML induced more luciferase activity relative to negative controls or the AD-MH1-ML construct (Fig. 5F), consistent with our co-immunoprecipitation data. These data further suggest that a physical interaction is formed between ARA55 and the MH2-ML region of Smad3 in live cells rather than only after cells are lysed.

ARA55 C Terminus Is Critical for Inhibition of Smad3 Activity—The binding of Smad3 and ARA55 could occur through ARA55 LD motifs or LIM domains, since both domains were reported to serve as protein-protein interaction interfaces. To define the region of ARA55 essential for the inhibition of Smad3 activity, we made Myc-tagged ARA55 truncations named A2–A8 (Fig. 6A). In co-IP experiments using HEK293 cells, all of the ARA55 truncations with the exception of A5 that do not contain LIM4 failed to bind to Smad3 (Fig. 6B). This was consistent with our co-immunoprecipitation data. Among these N-terminal truncations, A3 and A4 appeared to be the strongest inhibitors, which may have resulted from disruption of their nuclear export signal sequence, leading to nuclear accumulation of ARA55 (76). Unexpectedly, A5, which binds to Smad3 effectively (Fig. 6B), only had a partial inhibitory effect (Fig. 6C). A recent publication (77) showed that LIM4 domain may function as a nuclear localization signal. Thus, the lack of LIM4 in A5 possibly weakens the nuclear targeting ability of ARA55, causing its inefficiency to repress CA-Smad3 activity. Overall, these results imply that this inhibition occurs in the nucleus and that the translocation of CA-Smad3 is not affected by ARA55, consistent with our unpublished data.2 Our data showing that ARA55 associates with Smad3 are similar to a recent report (73), which also suggests that a physical interaction occurs between these two proteins, albeit in different cell lines and co-IP conditions (73).

Smad3 is critical for mediating TGF-β-induced apoptosis and growth suppression as well as the ability of TGF-β to function as a tumor promoter or tumor suppressor (2, 78–81). The possibility that ARA55 interferes with TGF-β-mediated apoptosis, thus contributing to carcinogenesis of the prostate, conforms to a role of ARA55 as an AR co-activator. On the other hand, ARA55 may function to reverse the ability of TGF-β to promote tumor progression and metastasis (2), consistent with the negative role of ARA55 in integrin signaling (54, 55).

Although our data in this study establish a structural basis for the physical interaction of ARA55 with Smad3 and for the ability of ARA55 to suppress transcriptional response of Smad3, the mechanism by which the interaction between ARA55 and Smad3 intercepts TGF-β responses remains to be defined. This LIM domain protein may disrupt the dimerization of Smad3 with itself or with Smad4 or interfere with the interaction of Smad3 with other common transcription factors or with SBE. Our preliminary data suggest that nuclear translocation of Smad3 by TGF-β is not blocked by ARA55 (data not shown). In contrast to our initial speculation that ARA55 may function through AR to intercept TGF-β responses, our data presented here suggest that ligand bound-AR is not involved in the inhibition of TGF-β responses by ARA55, at least in NRP-
154 cells. Aside from no detection of AR in NRP-154 cells (59), co-IP experiments indicate that ARA55 does not associate to AR (even when Smad3 is overexpressed) when co-transfected cells are cultured in medium containing 10% FBS, whereas the addition of DHT to this medium promoted the physical interaction AR with ARA55 (data not shown). Therefore, in the absence of exogenous androgens, culture medium containing 5% FBS or charcoal-stripped serum would probably not promote the association of endogenous AR to ARA55 to mediate suppression of Smad3 activity by ARA55.

In summary, this is the first report demonstrating that an AR co-regulator selectively blocks TGF-β signaling in prostatic cells, which occurs through a well defined physical interaction with Smad3 at the MH2-ML region. This provides new insight on the cooperative roles of AR with ARA55 or with potentially other AR-binding proteins as regulators of TGF-β signaling.

![Graph and Image Description](http://www.jbc.org/)

**Fig. 5.** Physical interaction between ARA55 and Smad3. A, ARA55 associates with Smad3 and Smad4 but not with Smad2. HEK293 cells, plated in 6-well dishes in 2 ml of DMEM/F-12 + 5% FBS, were co-transfected with 1 µg of pcDNA3-Myc-ARA55 or pcDNA3-Myc-paxillin and 1 µg of expression vectors for FLAG-Smads 2, 3, or 4 or the empty control vector. Cell lysates were immunoprecipitated with anti-FLAG IgG1 monoclonal antibody (M2), and both the immunoprecipitated proteins and input lysates were immunoblotted with rabbit anti-Myc IgG. B, the same co-IP experiment was performed as in A except that cells were transfected with pcDNA3-Myc-WT-Smad3 and pcDNA3-FLAG-ARA55. C, GST pull-down assay. HEK293 cells transfected with either pcDNA3 or pcDNA3-Myc-ARA55 were harvested with cold RIPA buffer. Cell lysates were incubated overnight with GST or GST-Smad3 in the presence of glutathione-Sepharose resin in cold RIPA buffer. The eluate was immunoblotted (IB) with anti-Myc antibody. D, diagram of Smad3 truncations with FLAG tag. From top to bottom, Smad3 full length, MH1-ML, MH1, MH2-ML, and MH2. N, N terminus; C, C terminus. E, MH2 domain of Smad3 is required for the interaction between ARA55 and Smad3. Smad3 truncations described in C were used for co-IP experiment performed in the same way as those of A. Mammalian two-hybrid assay confirms the interaction of ARA55 with Smad3 (F). NRP-154 cells were co-transfected with the constructs encoding BD-ARA55, AD-MH1-ML, or AD-MH2-ML and Gal4-luciferase reporter. AD-MH1-ML, NFkB AD-Smad3 MH1-ML region (0.4 µg/well); AD-MH2-ML, NFκB AD-Smad3 MH2-ML region (0.4 µg/well); BD-ARA55, Gal4 BD-ARA55 (0.4 µg/well); and Gal4-luciferase reporter (0.2 µg/well). Luciferase values in F represent the average of triplicate determinations ± S.E. All of the data shown are representative of three independent experiments.
the current model, Smad3, AR, and ARA55 may form a three-way relay connecting major signaling pathways that control cell growth, death, and carcinogenesis of the prostate. Therefore, further effort on understanding the functional interactions among these proteins may have therapeutic potential for prostate cancer.

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FIG. 6. The C terminus of ARA55 plays a major role in its inhibitory effect on CA-Smad3. A, diagram of Myc-tagged ARA55 truncation constructs (in pcDNA3 vector). B, interaction between WT-Smad3 and ARA55 truncations. HEK293 cells were co-transfected with 1 μg/well of each of pcDNA3-Myc-ARA55 truncation vectors and 1 μg/well pcDNA3-FLAG-Smad3 or the pcDNA3 control. The samples were immunoprecipitated and tested as described previously (31). C, effects of ARA55 truncations on CA-Smad3-induced SBE4 activity in NRP-154 cells. NRP-154 cells were co-transfected with 0.4 μg of SBE4_luciferase reporter, 12.5 ng of pcMV-RL, ±0.1 μg of pcDNA3-CA-Smad3, and ±0.5 μg pcDNA3-Myc-ARA55 or pcDNA3-Myc containing various truncations of ARA55. Luciferase values in C represent the average of triplicate determinations ± S.E. All of the data shown are representative of three independent experiments.
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