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Androgen receptor auto-regulates its expression by a negative feedback loop through upregulation of IFI16 protein

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Abstract **Expression of androgen receptor (AR) in prostate epithelial cells is thought to regulate cell proliferation, differentiation, and survival. However, the molecular mechanisms remain unclear. We report that re-expression of AR in PC-3 human prostate cancer cell line resulted in upregulation of IFI16 protein, a negative regulator of cell growth. We found that the IFI16 protein bound to AR in a ligand-dependent manner and the DNA-binding domain (DBD) of the AR was sufficient to bind IFI16. Furthermore, re-expression of IFI16 protein in LNCaP prostate cancer cells, which do not express IFI16 protein, resulted in downregulation of AR expression and an inhibition of the expression of AR target genes. Our observations identify a role for IFI16 protein in AR-mediated functions.**

Keywords: Prostate cancer; Androgen receptor; Interferon; IFI16

1. Introduction

Studies using animal and cell model systems have indicated that the expression of androgen receptor (AR) in prostate epithelial cells regulates cell proliferation, differentiation, and survival [1–3]. Furthermore, embryonic prostate epithelial cells from Rb-null mice were shown to express increased levels of the AR protein [4], indicating that the Rb protein negatively regulates the expression of AR. Importantly, mutations in both *Rb* [5] and *AR* [3,6] genes have been reported during the development of human prostate cancers.

Androgens activate the transcription of a set of interferon (IFN)-activatable genes in cultured rat ventral prostate epithelial cells [7]. Moreover, our recent study has revealed that treatment of normal human prostate stromal or epithelial cells with interferons (α, β, and γ) results in increases in AR protein levels and stimulation of AR-mediated transcription of AR-responsive reporter genes [8]. Interestingly, the IFN-activatable RNaseL protein binds to AR in a ligand-dependent manner and the expression of activated AR renders cells insensitive to the IFN treatment [9]. These studies suggest cross-talks between androgen signaling and IFN signaling in prostate epithelial and stromal cells. However, androgen and interferon effector proteins that mediate the growth-regulatory functions in prostate cells remain to be identified.

One family of interferon (IFN)-activatable genes is the 200-gene family [10]. The family includes mouse (for example, *Ifi202a*, *Ifi202b*, *Ifi203*, and *Ifi204*) and human genes (for example, *IFI16*, *MNDA*, and *AIM2*) that encode structurally related proteins (the p200-family proteins) [10,11]. Increased expression of p200-family proteins, such as p202 [11,12] and IFI16 [13,14], is known to inhibit cell cycle progression and modulate apoptosis [15]. Moreover, the p200-family proteins function as scaffold proteins and their binding to transcription factors is known to modulate the transcription of genes. Consistent with a role for IFI16 protein as a scaffold protein, IFI16 protein binds to p53 [16], pRb [13], E2F1 [13], and BRCA1 [15]. Furthermore, binding of IFI16 to Rb and E2F1 is correlated with inhibition of E2F1-mediated transcription [13].

Treatment of a variety of cells with IFNs (α, β, or γ) has been shown to result in upregulation of IFI16 mRNA and protein [10,13,14]. Interestingly, immortalization of normal human fibroblasts with SV40 large T antigen, which is known to inactivate both Rb and p53 tumor suppressors, resulted in downregulation of IFI16 expression [14], raising the possibility that the Rb and/or p53 pathways positively regulate the expression of IFI16. Consistent with a role of IFI16 protein in the Rb and p53 cell growth suppression pathways, the increased expression of IFI16 protein in cultured normal human fibroblasts [14] and prostate epithelial cells [13] was associated with cellular senescence. Moreover, knockdown of IFI16 expression in human diploid fibroblasts inhibited p53-mediated transcription, downregulated $p21^{CIP1}$ expression, and extended the proliferation potential of cells [14]. Consistent with a potential role for IFI16 in cellular senescence-associated cell cycle arrest, the expression of IFI16 protein was either very low or it was not detected in immortalized human fibroblast cell lines [14] and most prostate cancer cell lines tested [13]. Significantly, forced expression of IFI16 in prostate cancer cell lines (LNCaP, PC-3, and DU-145) inhibits cell proliferation [13], which in PC-3 cell line was associated with upregulation of $p21^{CIP1}$ expression and a senescence-like phenotype [13]. However, it remains unknown how the expression of IFI16 is regulated in prostate epithelial cells.

Here, we report that re-expression of AR in PC-3 cells resulted in upregulation of IFI16 mRNA and protein. We also found that increased expression of IFI16 in LNCaP prostate cancer cells resulted in downregulation of AR expression and an inhibition of AR-mediated transcription of target genes.

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2. Materials and methods

2.1. Cell culture and transfections

PC-3 and LNCaP prostate cancer cell lines were maintained in DMEM culture medium (Invitrogen Life Technologies) supplemented with 10% (v/v) fetal bovine serum and antibiotics. If so indicated, cells were either cultured in basal medium (without phenol red; Cambrex, Walkersville, MD) or RPMI 1640 (without phenol red; Invitrogen) supplemented with 10% charcoal-stripped fetal bovine serum (CS-FBS; Sigma). When indicated cells were cultured in the presence of the indicated concentration of androgen receptor ligand dihydrotestosterone (DHT). PC-3 cells were transfected with an empty vector (pCMV) or plasmid encoding human AR (pCMV-hAR; plasmid generously provided by Dr. N. Weigel, Baylor College of Medicine, Houston, TX). The transfected cells were selected in 500 μg/ml of G418 for about two weeks and >100 G418-resistant colonies were pooled for further analysis.

HEK-293 or LNCaP cells were nucleofected with pCMV-hAR, pCDNA3-IFI16B, or an empty vector (pCMV) plasmid using Nucleofector-II device (Amaxa Biosystems, Germany). HEK-293 and LNCaP cells were nucleofected as suggested by the supplier using nucleofection kit VCA-1003 (program Q-001) and VCA-1001 (program T-009), respectively. If so indicated, 24–48 h after nucleofections of cells, cells were grown in RPMI 1640 medium (without phenol red; Invitrogen) supplemented with 10% CS-FBS and DHT (10 nM).

2.2. RT-PCR

Total RNA was isolated from the indicated cells using TRIzol reagent (Invitrogen) as suggested by the supplier. Isolated total RNA was first treated with DNase to eliminate contamination of any chromosomal DNA. The treated RNA (0.5–1 μg) was subjected to One-Step RT-PCR, using a pair of primer specific to *IFI16* [14], and a kit from Invitrogen Life Technologies, as suggested by the supplier.

2.3. Immunoblotting

Total cell lysates were subjected to immunoblotting as described previously [13]. Monoclonal mouse antibodies to IFI16 (sc-8023) and AR (sc-7305) were purchased from Santa Cruz Biotech. Inc.

2.4. Immunoprecipitations and GST-pull-down assays

Immunoprecipitations using a monoclonal antibody (sc-8023; Santa Cruz) to IFI16 protein, using a monoclonal (sc-7305; Santa Cruz) or a polyclonal antibody to AR (sc-815) were performed as described previously [17]. Plasmids encoding GST-hAR (1–562), GST-hAR (544–634), and GST-hAR (624–919) were generously provided by Dr. F.S. French (University of North Carolina, Chapel Hill, NC). Plasmid encoding GST-IFI16 has been described [13]. GST-fusion proteins were produced, purified, and used as described previously [18].

2.5. Reporter assays

Dr. Alexander Chlenski (Northwestern University, Chicago) generously provided the pGL-AR3.5-luc (indicated as AR3.5-luc) reporter plasmid [19]. We have amplified 1.68 kb genomic fragment from the 5′-regulatory region of the *IFI16*-gene from a human genomic DNA library (purchased from Clontech, Palo Alto, CA). We have sequenced this 1.68 kb genomic fragment, identified a potential transcription initiation site, and analyzed for potential DNA binding sites for various transcription factors. Moreover, we have linked this 1.68 kb regulatory region of *IFI16* to the luciferase reporter gene in pGL3 basic plasmid (without any promoter and enhancer sequences), resulting in IFI16-luc-reporter plasmid. The activity of IFI16-luc-reporter was stimulated >2-fold in PC-3 and DU-145 cells by IFN-α or IFN-γ treatment for 24 h. Luciferase reporter assays were performed as described previously [14].

3. Results

We have reported previously that the expression of IFI16 mRNA and protein is either not detectable or very low in most prostate cancer cell lines tested [13]. Additionally, we found that immortalization of normal human diploid fibroblasts with SV40 large T antigen, which inactivates both retinoblastoma protein and p53 tumor suppressor [20], results in reduced expression of IFI16 [14]. Because LNCaP prostate cancer cells do not express detectable levels of IFI16 mRNA and protein [13], to investigate whether AR could regulate the expression of IFI16 in human prostate cancer cells, we chose the approach involving re-expression of AR in PC-3 human prostate cancer cell line.

3.1. Re-expression of AR in PC-3 cells activates the transcription of the IFI16 gene

We have noted that PC-3 prostate cancer cells express detectable levels of IFI16 mRNA and protein [13]. However, these cells are reported to lack the expression of androgen receptor [6]. Therefore, to determine whether androgens through AR could regulate the expression of IFI16 in PC-3 prostate cancer cells, we generated a stable cell line (designated as PC-AR) from a large pool of cell clones selected after transfection of the plasmid (pCMV-AR) that allow the constitutive expression of human androgen receptor. As a control, we also generated a cell line from a large pool of vector (pCMV)-transfected cells (designated as PC-V). As shown in Fig. 1A, PC-AR cells expressed detectable levels of AR protein as compared to PC-V cells (compare lane 4 with 2) and addition of androgen receptor ligand DHT (1 nM) in the culture medium resulted in further increases in AR protein levels (compare lane 3 with 4). Importantly, treatment of the PC-AR cells with increasing concentrations of DHT for 24 h (Fig. 1B) or a fixed concentration of DHT (10 nM) for the indicated duration of time (Fig. 1C) resulted in increases in IFI16 protein levels. Moreover, we noted that the incubation of PC-AR cells with DHT (1 nM) for 24 h resulted in accumulation (30–50%) of AR protein in the nucleus (data not shown). Significantly, levels of IFI16 mRNA were measurably higher in PC-AR than PC-V cells (Fig. 1D). Furthermore, a comparison of the activity of IFI16-luc-reporter between the PC-V and PC-AR cells indicated that the activity of the reporter was about 2.5-fold higher in the PC-AR than PC-V cells (Fig. 1E). Together, these observations indicated that re-expression of AR in PC-3 cells resulted in transcriptional activation of the *IFI16* gene.

3.2. IFI16 protein associates with AR

Increased expression of the p200-family proteins and their demonstrated binding to a number of transcription factors is known to modulate the transcription of their target genes [11,12]. Therefore, we explored whether IFI16 protein could bind to AR. As shown in Fig. 2A, AR was detected in immunoprecipitates using a monoclonal antibody to IFI16 protein in immunoprecipitation-immunoblotting assays (IP-IB assays) from extracts derived from HEK-293 cells (we chose these cells because they do not express detectable levels of AR, however, they express low basal levels of IFI16 protein; data not shown) that were transfected with plasmids encoding AR and IFI16, but not AR or IFI16 alone. Furthermore, treatment of HEK-293 cells with androgen-ligand DHT resulted in measurable increases in association between IFI16 and AR protein in IP-IB assays (Fig. 2B), indicating that interactions between IFI16 and AR may depend on the AR ligand DHT.

LNCaP cells express a mutant form (T877A) of the AR protein [3,6], but do not express IFI16 protein [13]. Therefore, to

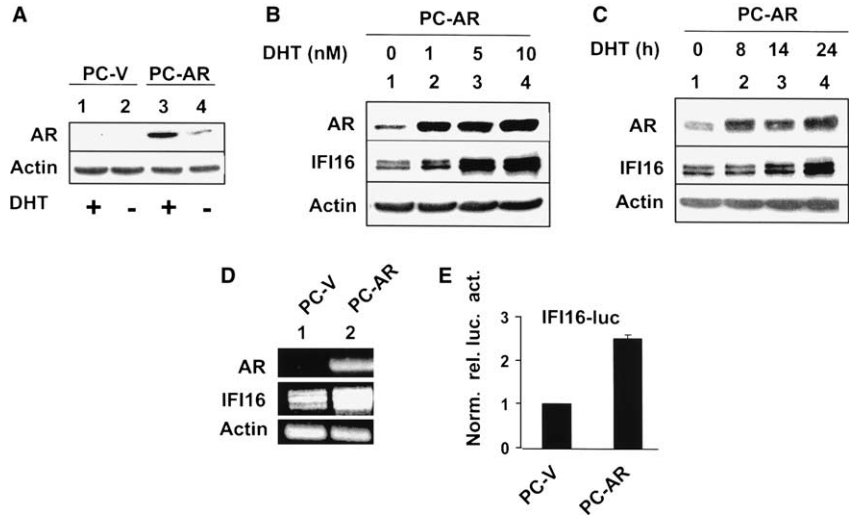

Fig. 1. Re-expression of AR protein in PC-3 cells results in upregulation of IFI16 expression. (A) Extracts prepared from vector (PC-V; lanes 1 and 2) or AR (PC-AR; lanes 3 and 4) transfected cells, either left untreated (lanes 2 and 4) or treated with DHT (1 nM for 24 h; lanes 1 and 3) were analyzed by immunoblotting using antibodies specific to the indicated proteins. (B) Extracts prepared from PC-AR cells cultured (for 24 h) in medium supplemented with 10% CS-FBS (lane 1) or 10% CS-FBS and the indicated concentration of DHT were analyzed by immunoblotting using antibodies specific to the indicated protein. (C) Extracts prepared from PC-AR cells cultured in medium supplemented with 10% CS-FBS and 10 nM DHT for the indicated time (h) were analyzed by immunoblotting using antibodies specific to the indicated protein. (D) Total RNA isolated from PC-V (lane 1) or PC-AR (lane 2) cells (cultured as described in panel C) was subjected to semi-quantitative RT-PCR using a pair of primer specific to the indicated gene. (E) Sub-confluent PC-V or PC-AR cells were transiently transfected with IFI16-luc-reporter plasmid as described in Section 2. The normalized relative activity of the IFI16-luc-reporter is shown.

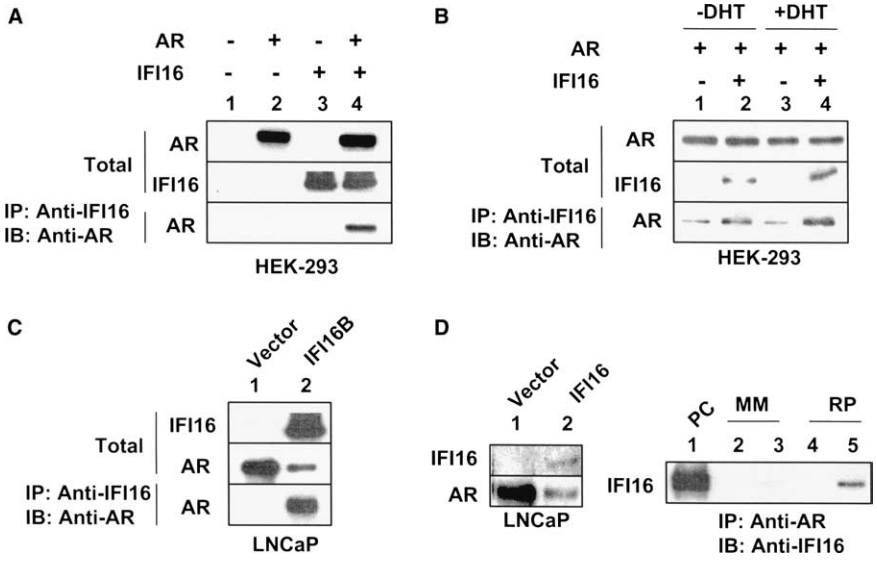

Fig. 2. IFI16 protein binds to AR. (A) Sub-confluent cultures of HEK-293 cells were nucleofected with equal amounts of an empty plasmid (pCMV; lane 1), plasmid pCDNA3-IFI16B encoding the IFI16 protein (lanes 3 and 4), or plasmid pCMV-AR encoding the AR protein (lanes 2 and 4). 24 h after nucleofections, cell lysates were subjected to immunoprecipitations using monoclonal antibody to IFI16 protein. Total cell lysates as well as the immunoprecipitates were analyzed by immunoblotting using antibodies specific to the indicated proteins. (B) Sub-confluent cultures of HEK-293 cells were nucleofected with equal amounts of pCDNA3-IFI16B plasmid encoding IFI16 protein (lanes 2 and 4), or pCMV-AR plasmid encoding AR protein (lanes 1–4). 24 h after nucleofections, cell were incubated with medium supplemented with CS-FBS or CS-FBS and 10 nM DHT for 24 h. Cell lysates were subjected to immunoprecipitations using antibodies to IFI16 protein. Total cell lysates as well as the immunoprecipitates were analyzed by immunoblotting using antibodies to the indicated proteins. (C) Sub-confluent cultures of LNCaP cells were nucleofected with equal amounts of pCDNA3 plasmid (vector; lane 1) or pCDNA3-IFI16B plasmid encoding IFI16 protein (lanes 2). 24 h after nucleofections, cell lysates were subjected to immunoprecipitations using monoclonal antibody to IFI16 protein. Total cell lysates as well as the immunoprecipitates were analyzed by immunoblotting using antibodies to the indicated proteins. (D) Sub-confluent cultures of LNCaP cells were nucleofected with equal amounts of pCDNA3 plasmid (vector; lane 1) or pCDNA3-IFI16B plasmid encoding IFI16 protein (lanes 2). 24 h after nucleofections, cell lysates were subjected to immunoprecipitations using either a mouse monoclonal (MM) or a rabbit polyclonal (RP) antibody to the AR protein. Total cell lysates (left panel) as well as the immunoprecipitates (right panel) were analyzed by immunoblotting using antibodies to the indicated proteins.

further examine interactions between IFI16 protein and AR in vivo, we restored the expression of IFI16 protein in LNCaP cells and performed IP-IB assays. As shown in Fig. 2C, AR protein was detected in IFI16 immunoprecipitates from extracts derived from LNCaP cells (cells grown in medium supplemented with 10 nM DHT) that were transfected with a

plasmid encoding IFI16 protein, but not an empty plasmid (compare lane 2 with 1). Conversely, IFI16 protein was detected in AR immunoprecipitates using the C-terminal rabbit polyclonal antibody (RP) to AR, but not a monoclonal antibody (MM) against the epitope comprising amino acids 299–315 (Fig. 2D, compare lane 5 with 3), from extracts derived from LNCaP cells that were transfected with a plasmid encoding IFI16 protein, but not an empty vector (Fig. 2D, right panel, compare lane 5 with 4). Together, these observations indicated that IFI16 protein associated with the endogenous mutant (T877A) AR protein in extracts derived from the LNCaP cells.

Association of IFI16 protein with AR in above experiments prompted us to localize IFI16 binding region(s) in AR protein. Therefore, we performed GST-pull-down assays in which we incubated extracts from PC-3 (these cells express detectable levels of IFI16 protein; Fig. 1) with glutathione–sepharose beads (Fig. 3A, lane 2) or beads loaded with the indicated GST-AR proteins. As shown in Fig. 3A and B, IFI16 in extracts derived from the PC-3 cells bound to AR in highly conserved DNA-binding domain (amino acids 544–634) and not in the N-terminal (amino acids 1–562) or the C-terminal (amino acids 624–919) region. Together, these observations indicated that IFI16 protein associated with AR in vitro and in vivo and a conserved DNA-binding domain (DBD) in AR is sufficient to bind IFI16 protein in vitro.

3.3. Re-expression of IFI16 in LNCaP cells downregulates the AR expression and inhibits AR-mediated transcription

Binding of IFI16 protein to AR in DNA-binding region in above experiments prompted us to test whether re-expression of IFI16 in LNCaP cells, which do not express IFI16 protein [13], has any effect on AR-mediated transcription. For this purpose, we nucleofected the LNCaP cells with increasing amounts of the pCDNA3-IFI16B plasmid that encodes the IFI16B protein or, as a negative control, an empty pCDNA3 plasmid, and analyzed the expression levels of IFI16, AR, and prostate specific antigen (PSA; a transcriptional target of the AR) mRNA levels by RT-PCR. As shown in Fig. 4A, re-expression of IFI16 in LNCaP cells resulted in measurable decreases in the levels of the AR mRNA and moderate decreases in PSA mRNA levels. Moreover, we noted that re-expression of IFI16B in LNCaP cells also resulted in decreases in AR protein levels (Fig. 4B) and 40–50% decrease in the activity of AR3.5-luc-reporter, the expression of which was driven by the 3.5-kb 5′-regulatory region of the *AR* gene [19]. Because increased expression of IFI16 protein in prostate cancer cell lines (including the LNCaP) inhibits cell proliferation [13], we compared the expression of AR target protein, the PSA and other growth-regulatory proteins between vector and IFI16 nucleofected LNCaP cells. As shown in Fig. 4C, re-expression of IFI16 protein in LNCaP cells resulted in appreciable decreases in levels of AR, PSA, Cyclin A, and Cyclin D1 protein levels. Interestingly, we noted an increase in p73, a member of the p53 protein family, protein levels and moderate decreases in E2F1 protein levels. Together, these observations suggested that re-expression of IFI16 in LNCaP cells inhibits cell proliferation, in part, by downregulating the expression of AR and by inhibiting the AR-mediated transcription of its target genes.

4. Discussion

The p200-family proteins are thought to function as scaffold proteins that assemble protein complexes regulating transcription of genes [13,14]. Therefore, the function of IFI16 protein in cell growth regulation depends on its ability to bind with other proteins and modulate their functions. To date, several cellular proteins have been known to bind IFI16 protein.

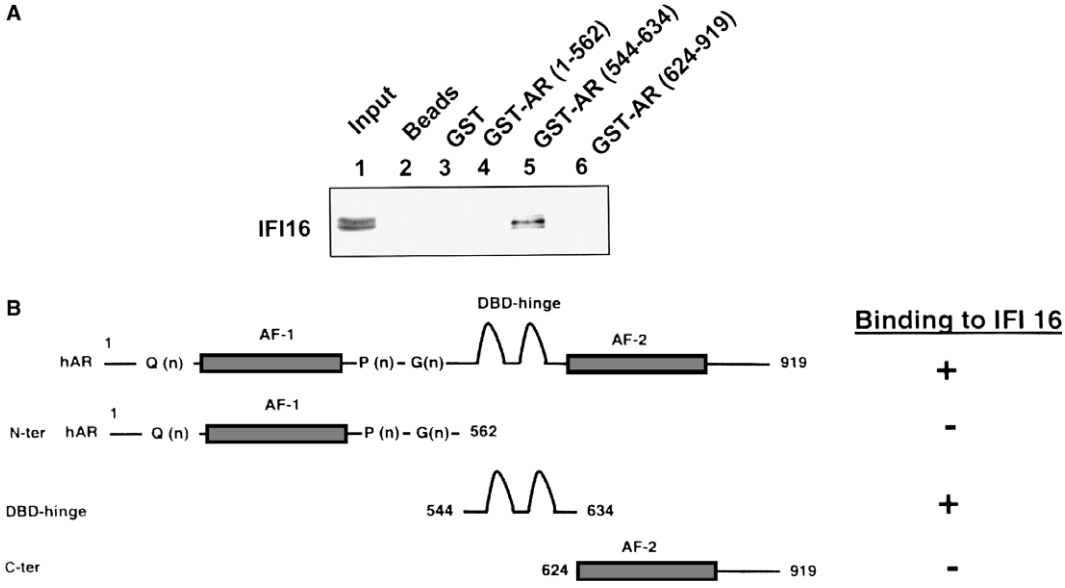

Fig. 3. IFI16 binds to AR in the DNA binding domain. (A) Extracts from PC-3 cells were incubated with sepharose beads (lane 2) or beads loaded with the indicated GST-AR-fusion proteins. Bound cellular proteins were analyzed by immunoblotting using monoclonal antibody to IFI16. A fraction (2%) of total cell extracts was also run in lane 1. (B) Schematic presentation of various structural domains of human AR and their binding to IFI16 protein in assays shown in Fig. 1A–E.

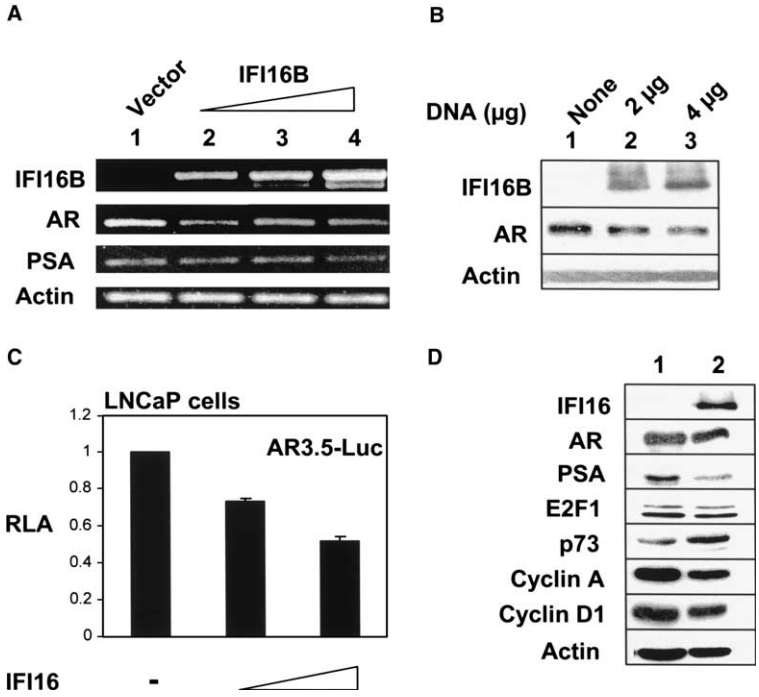

Fig. 4. Re-expression of IFI16 protein in LNCAP cells downregulates the expression of AR. (A) Sub-confluent cultures of LNCaP cells were nucleofected with pCDNA3 plasmid (vector; lane 1) or increasing amounts of pCDNA3-IFI16B plasmid. 24 h after nucleofections, total RNA was isolated from attached and floating cells. Equal amounts of total RNA was subjected to RT-PCR using a pair of primers specific to *IFI16*, human *AR*, *PSA*, or actin gene. (B) LNCaP cells were nucleofected as described in panel A, and 24 h after nucleofections total cell lysates were analyzed by immunoblotting using antibodies specific to the indicated proteins. (C) LNCaP cells were transiently transfected with AR3.5-luc-reporter and pRL-TK-reporter plasmids (in 5:1 ratio) along with the indicated expression plasmid using Superfect transfection agent (Qiagen). The amount of total transfected plasmid DNA was adjusted to 2 μg using an empty pCDNA3 plasmid. 45 h after transfections, the firefly luciferase and *Renilla* luciferase activities were determined. The normalized relative luciferase activity is shown. (D) Sub-confluent cultures of LNCaP cells were nucleofected with pCDNA3 (lane 1) or pCDNA3-IFI16B (lane 2) plasmid. 24 h after nucleofections, total cell lysates were analyzed by immunoblotting using antibodies specific to the indicated proteins.

These proteins include Rb and E2F [13], BRCA1 [15], and p53 [16]. Importantly, cell growth inhibition by IFI16, in part, depends on its ability to potentiate the Rb/E2F-mediated transcriptional repression [13] and to upregulate the expression of $p21^{CIP1}$ in p53-dependent [14] and independent manner [13].

Genetic alterations in the p16/Rb pathway are required for bypassing cellular senescence in human prostate epithelial cells [21]. Our observations that: (i) re-expression of AR in PC-3 cells results in upregulation of IFI16 (Fig. 1); (ii) IFI16 protein binds to AR in DNA-binding region (Fig. 3); and (iii) re-expression of IFI16 protein in LNCaP cells results in downregulation of AR expression and inhibition of the expression of AR target proteins (Fig. 4), provide support for the idea that the upregulation IFI16 by AR in prostate epithelial cells is a part of an important negative feedback loop, which regulates the expression of AR and AR-mediated functions.

Increased AR mRNA levels have been detected in senescent normal human prostate epithelial cells [22]. Because IFI16 levels also increase in senescent normal human prostate epithelial cells [13], further work is in progress to determine whether the AR-mediated upregulation of IFI16 in prostate epithelial cells contributes to senescence-associated cell growth arrest.

Re-expression of AR in PC-3 cells was found to inhibit cell proliferation [23,24]. Consistent with these reports, we also noted that re-expression of AR in PC-3 cells (PC-AR cells; Fig. 1) resulted in retardation of cell proliferation as compared to vector transfected cells (data not shown). Because increased expression of IFI16 protein in PC-3 cells inhibits cell proliferation [13], our observations provide support for the idea that AR-mediated upregulation of IFI16 in PC-3 cells contributes to inhibition of cell growth.

Mutations in p53 gene are thought to be a late event during the development of prostate cancer [1] and loss of p53 function in prostate epithelial cells is known to confer a hormone resistance phenotype [25]. Because IFI16 binds to p53 and potentiates the p53-mediated transcription [14,16], our observations raise the possibility that reduced expression of IFI16 in prostate cancer cells may also contribute to hormone resistant phenotype. Consistent with this idea, downregulation of $p21^{CIP1}$ expression in prostate cancer cells is linked to androgen independence [26].

We have noted that the 1.6 kb 5′-regulatory region of the *IFI16* gene, which we have analyzed for the presence of potential AR binding sites, does not contain the well-known (5′-GGA/TACANNNTGTTCT-3′) AR-responsive elements (AREs). However, it does contain several 6-bp-long (5′-TGTTCT-3′) ARE's core sequences that are flanked by the AP-1 DNA binding sites. Because the AR can activate the transcription of genes through binding to the transcription factor c-Jun [27], and AP-1 (c-Jun and c-Fos) is known to activate the transcription of *IFI16* gene [28], additional work will be needed to determine whether AR could activate the transcription of *IFI16* gene in prostate epithelial cells through the c-Jun/AP-1 DNA-binding sites.

Proteins containing the LXXLL-like motifs bind to AR in the hormone-binding domain and regulate its transcriptional activity [29]. We have noted that IFI16 protein contains at least five LXXLL-like motifs (^{7}NxxLL^{11}, ^{25}VXXLL^{29}, ^{33}LXXNL^{37}, ^{62}LXXLI^{66}, and ^{75}LXXLA^{79}; the numbers indicate the amino acid residues in IFI16 protein) in the N-terminus. Additionally, the IFI16 protein contains two partially conserved repeats of 200-amino acid residues (also called HIN-200 domain), which are found in all p200-family proteins and are shown to participate in protein–protein interactions [11,12]. Because IFI16 protein bound to AR in DNA-binding domain in GST-pull-down assays (Fig. 3) and not in the hormone binding domain, further work is in progress to identify the AR binding region(s) in the IFI16 protein.

In summary, our observations provide evidence for the role of IFI16 in AR-mediated regulation of prostate epithelial cell growth. These observations will serve as basis to: (i) elucidate the molecular mechanisms by which IFI16 contributes to the regulation of prostate epithelial cell growth; (ii) determine how loss of IFI16 expression in normal prostate epithelial cells contributes to the initiation and progression of prostate cancer through deregulation of AR expression.

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