FEBS Letters 580 (2006) 1659–1664

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

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Received 21 December 2005; revised 6 February 2006; accepted 8 February 2006

Available online 17 February 2006

Edited by Varda Rotter

**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.**
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*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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doi:10.1016/j.febslet.2006.02.015

## 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

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