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Androgen receptor primes prostate cancer cells to apoptosis through down-regulation of basal p21 expression

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ABSTRACT

The androgen receptor (AR) for the male hormone androgen plays an important role in regulation of cell survival or death depending on the nature of cellular context and extracellular stimuli. The pro-survival function of AR is mediated mainly by transcriptional regulation of its target genes. By contrast, the pro-death function of AR can be transcription-dependent or -independent, although the underlying mechanism of the latter is incompletely understood. Here we report that, in androgen-independent prostate cancer cells, AR promotes UV-induced apoptosis through down-regulation of basal expression of p21 independently of its transcriptional activity. Down-regulation of basal p21 expression depends on AR N-terminal interacting protein PIRH2, an E3 ligase for proteasomal degradation of p53. Silencing of PIRH2 up-regulates p53, which in turn activates p21 transcription. Consistent with this, knockdown of PIRH2 suppresses UV-induced AR-dependent apoptosis. Our data suggest that AR primes androgen-independent prostate cancer cells to DNA damage-induced apoptosis through the PIRH2-p53-p21 axis.

1. Introduction

Androgen receptor (AR) is a member of the steroid hormone receptor superfamily [1,2]. Like other steroid hormone receptors, AR has three known functional domains: the N-terminal transactivation domain, the C-terminal DNA-binding domain (DBD), and the ligand-binding domain (LBD) [2]. There are several regulatory elements in the N-terminal transactivation domain, including AF1 (activation function 1) and post-translational modification sites for phosphorylation and ubiquitination [3–5]. The AR N-terminal transactivation domain also interacts with dozens of proteins [6] including PIRH2 (p53-induced RING-H2 protein; also known as ARNIP, AR N-terminal interacting protein) [7,8]. However, the biological function of the interaction between AR and PIRH2 is incompletely understood.

The androgen-AR signaling pathway plays a critical role in regulation of cell death [9–11]. While AR exerts its pro-survival and pro-growth functions through transcriptional regulation of its target genes [12,13], it also has pro-death activity, especially in late stage androgen-independent prostate cancer [14,15]. Previously, we have shown that AR can augment UV-induced cell death through promotion of Bax mitochondrial translocation or generation of apoptotic AR proteolytic fragments [14,16], demonstrating that AR is able to regulate death independent of its transcriptional activity.

The AR N-terminal interacting protein PIRH2 interacts with more than 20 proteins and exerts its functions through various mechanisms [8,17]. It has been reported that PIRH2 can inhibit AR N- and C-terminal interactions [8]. PIRH2 also enhances recruitment of AR to the promoter of the prostate specific antigen (PSA) gene, or promotes degradation of the AR co-repressor, HDAC1 [18,19]. Furthermore, PIRH2 functions as an E3 ligase for a group of signaling molecules involved in DNA damage response, including p53, p73, and p27 [7,17,20,21].

The cell cycle inhibitor p21 is a target gene of p53 [22–24], and its level of expression often determines the sensitivity of cells to genotoxic stresses such as UV irradiation [25–27]. Different E3 ligases, such as SCF and CRL4, have been shown to be involved in p21 degradation, [28,29]. Here we show that AR down-regulates basal p21 expression through the PIRH2-p53-p21 axis in androgen-independent prostate cancer cells, thereby priming the cells to UV-induced apoptosis.

2. Materials and methods

2.1. Reagents and cell culture

Antibodies against p21 (H164) and AR (H280) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against β-actin was from Millipore (Billerica, MA). Antibody against PIRH2

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was from Abcam (Cambridge, MA). The fluorogenic caspase-3 substrate DEVD-AFC (carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin), protein synthesis inhibitor cycloheximide (CHX), and proteasomal inhibitor MG-132 were from Calbiochem (La Jolla, CA). Human prostate cancer cell LNCaP subline 104-R cells and AR knockdown 104-R (AR siRNA) cells were generated and maintained as described previously [14]. Both cell lines were cultured in Dulbecco's Modified Eagle medium (DMEM) with 10% dextran-coated, charcoal-stripped fetal bovine serum [9].

2.2. Immunoblot analysis

Cells were harvested and lysed in NP-40 buffer (145 mM NaCl, 5 mM $MgCl_2$, 20 mM HEPES, 1 mM EGTA, 1 mM EDTA, 10 mM DL-dithothreitol (DTT), 0.2% Nonidet P40) with a protease inhibitor cocktail (100 μM PMSF, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin, 1 mM Benzamidine). Equal amounts of cell lysates (60 μg) were subjected to SDS–PAGE and transferred onto a PVDF membrane. The membrane was blocked in 3% fat-free milk or 3% BSA for 1 h and incubated sequentially with the primary antibody overnight at 4 °C. The target proteins were detected using Super-Signal chemiluminescence reagents (Pierce Biotechnology, Inc., Rockford, IL).

2.3. Caspase-3 activity assay

Assays of Caspase-3 activity were performed as previously described [30]. Briefly, cell lysate (40 μg) in buffer A (25 mM HEPES, pH 7.4, 5 mM EDTA, 2 mM DTT, 0.1% Chaps) was incubated at 37 °C for 1 h with fluorogenic caspase-3 substrate DEVD-AFC in the dark. Fluorogenic intensities were then monitored using a Gemini EM microplate spectrofluorometer (Molecular Devices) at 360 nm excitation and 445 nm emission.

2.4. RNA interference

All siRNA duplexes were designed and obtained from Dharmacon Research. The siRNA targeting sequence for AR is 5′-CTTCGACCATTTCTGACAA-3′; for PIRH2, 5′-CCAACAGACTTGTGAAGAA-3′; for p53, 5′-AGACCTATGGAAACTACTT-3′; and that for luciferase, 5′-CGUACGCGGAAUACUUCGA-3′. The siRNAs were transfected into cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions; after 48 h, cells were harvested for further experimentation.

2.5. Lentivirus encoding siRNA

The lentiviral RNA interference expression vector pLKO.1, VSVG envelope protein vector pCMV-VSV-G, and lentiviral packaging vector pCMV-dR8.2 dvpr were from Addgene [31]. For each target gene, a pair of complementary 64-base hairpin oligonucleotides, each containing a palindromic 19-base pair of targeted siRNA sequences in inverted repeat orientation, were synthesized (Integrated DNA Technologies). After annealing, the 64-mer duplex was inserted into AgeI- and EcoRI- digested pLKO.1. The siRNA/pLKO.1 constructs were then transfected into HEK293T cells with pCMV-VSV-G and pCMV-dR8.2 dvpr, and viral supernatants were collected 48 h post transfection. These supernatants were used immediately to infect target cells, which were selected for three weeks with puromycin to remove uninfected cells prior to experimentation.

2.6. Analytical tools and statistics

The relative densities of p21 protein signals in immunoblots were measured and analyzed using the Image Processing and Analysis in Java (Image J) program (http://rsbweb.nih.gov/ij/). Statistical analysis for comparisons of caspase-3 activities and the relative densities of p21 protein were performed using the two-tailed Student's *t* test. *p*-values less than 0.05 were considered statistically significant.

3. Results

3.1. UV induces p21 degradation in an AR-dependent but androgen-independent manner

Previously, we have showed that AR can sensitize UV-induced AR positive prostate cancer cell death in an androgen-independent manner, by promoting Bax mitochondrial translocation or generation of pro-apoptotic AR N-terminal fragments [14,16]. In searching for additional downstream effector(s) that may mediate androgen-independent pro-death activity of AR, we noticed that the protein level of p21, which is an AR target gene, was significantly reduced in androgen-independent AR-positive 104-R cells 90 min after UV stimulation (Fig. 1A). The reduction of p21 proteins was inhibited by the proteasomal inhibitor MG-132 (Fig. 1B), but not influenced by androgen (Fig. 1A). These results demonstrated that p21 underwent proteasomal degradation upon UV stimulation, consistent with the previous reports [27]. Interestingly, UV-induced apoptotic cell death was also inhibited by MG-132 but not by androgen (Fig. 1C) [13]. The degradation of p21 was not a consequence of cell death, as it occurred within 1 h following UV irradiation, while the onset of the cell death was detected only after 4–8 h (Fig. 1C). Thus, proteasomal degradation of p21 proteins may contribute to UV-induced apoptosis in AR-positive prostate cancer cells.

3.2. AR regulates basal p21 expression independently of its transcription activity

To determine whether the degradation of p21 is regulated by AR, 104-R cells were stably transfected with control siRNA (siCtrl)

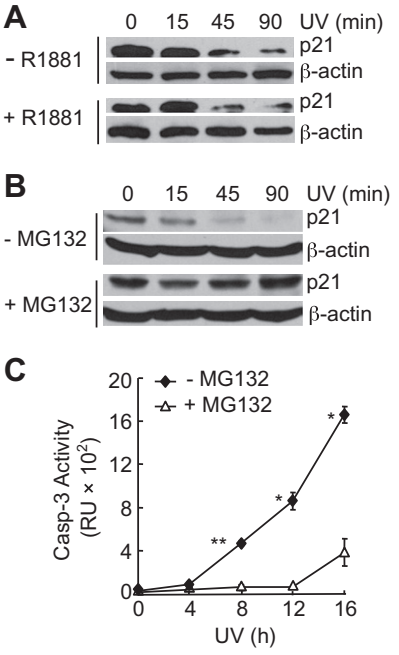

Fig. 1. UV-induced degradation of p21 protein in AR-positive 104-R cells. (A) 104-R cells were treated with or without a synthetic androgen, R1881 (2 nM) for 2 h followed by UV irradiation (100 J/m^2). The p21 protein levels were analyzed by immunobloting with anti-p21 antibody. (B and C) 104-R cells were treated with or without proteasome inhibitor MG-132 (10 μM) for 2 h. The p21 protein levels were analyzed by immunoblotting (B), as described in (A). Caspase activity was measured using fluorogenic substrate Ac-DEVD-AFC (C). $^*p < 0.05$, $^{**}p < 0.01$.