Inhibition of Stat5a/b Enhances Proteasomal Degradation of Androgen Receptor Liganded by Antiandrogens in Prostate Cancer

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Abstract

Although poorly understood, androgen receptor (AR) signaling is sustained despite treatment of prostate cancer with antiandrogens and potentially underlies development of incurable castrate-resistant prostate cancer. However, therapies targeting the AR signaling axis eventually fail when prostate cancer progresses to the castrate-resistant stage. Stat5a/b, a candidate therapeutic target protein in prostate cancer, synergizes with AR to reciprocally enhance the signaling of both proteins. In this work, we demonstrate that Stat5a/b sequesters antiandrogen-ligated (MDV3100, bicalutamide, flutamide) AR in prostate cancer cells and protects it against proteasomal degradation in prostate cancer. Active Stat5a/b increased nuclear levels of both unliganded and antiandrogen-ligated AR, as demonstrated in prostate cancer cell lines, xenograft tumors, and clinical patient-derived prostate cancer samples. Physical interaction between Stat5a/b and AR in prostate cancer cells was mediated by the DNA-binding domain of Stat5a/b and the N-terminal domain of AR. Moreover, active Stat5a/b increased AR occupancy of the prostate-specific antigen promoter and AR-regulated gene expression in prostate cancer cells. Mechanistically, both Stat5a/b genetic knockdown and antiandrogen treatment induced proteasomal degradation of AR in prostate cancer cells, with combined inhibition of Stat5a/b and AR leading to maximal loss of AR protein and prostate cancer cell viability. Our results indicate that therapeutic targeting of AR in prostate cancer using antiandrogens may be substantially improved by targeting of Stat5a/b. Mol Cancer Ther; 14(3): 1–14. ©2014 AACR.

Introduction

Although curative therapies exist for organ-confined or locally advanced prostate cancer, treatment options are limited for metastatic castrate-resistant prostate cancer (CRPC). Progression to CRPC is defined by resistance to androgen-deprivation therapy, which typically occurs less than 3 years after initiation of androgen-deprivation therapy (1–3). The transition from androgen-dependent prostate cancer to CRPC is not fully understood.

Androgen receptor (AR) signaling is known to be maintained despite low levels of circulatory androgens (1), which has been attributed to numerous mechanisms, including (i) AR gene amplification (4); (ii) AR ligand-binding domain mutations conferring ligand promiscuity (5, 6); (iii) intracrine androgen biosynthesis within prostate cancer cells from adrenal steroids and cholesterol (7, 8); (iv) ligand-independent, noncanonical AR transactivation by kinase signaling pathways (9, 10); and (v) upregulation of constitutively active, AR splice variants that do not require ligand to support prostate cancer growth (11, 12).

Most therapeutic strategies for prostate cancer are directed at the AR signaling axis, with established antiandrogens such as
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flutamide and bicalutamide being joined by recently U.S. Food and Drug Administration–approved agents such as the androgen biosynthesis inhibitor abiraterone acetate (Zytiga; refs. 13, 14) and the second-generation antiandrogen MDV3100 (enzalutamide, Xtandi; ref. 14). The first-generation antiandrogens function by competitive inhibition of androgen binding to AR (15, 16) and by altering coactivator and corepressor recruitment to induce formation of a transcriptionally inactive AR complex (16). The second-generation antiandrogen MDV3100 induces a distinct conformational change in AR, which impairs AR nuclear localization and DNA binding (17).

Signal transducer and activator of transcription 5a/b (Stat5a/b) provides a non-AR therapeutic target protein in prostate cancer (18–26). Transcription factor Stat5a/b is composed of two highly homologous isoforms, Stat5a and Stat5b, which display >90% amino acid identity and function as both signaling proteins and nuclear transcription factors. Activation of Stat5a/b occurs by phosphorylation of a conserved C-terminal residue by an upstream kinase, most commonly Jak2 in prostate cancer, which induces Stat5a/b dimerization, nuclear translocation, and targeting gene regulation (27, 28). Stat5a/b promotes growth of prostate cancer and tumor progression, critically sustaining viability of prostate cancer cells in vitro (18, 21) and xenograft tumor growth in vivo (19). Stat5a/b is active in 95% of clinical CRPCs (29), with the Stat5a/b gene locus amplified in 29% of distant CRPC metastases (30). In addition, high active Stat5a/b expression predicts early disease recurrence (24, 26) and prostate cancer–specific death (26), and promotes metastatic behavior of prostate cancer cells in vitro and in vivo (22), suggesting Stat5a/b involvement in clinical progression of prostate cancer. In further support of this concept, pharmacologic targeting of Stat5a/b signaling blocked growth of not only primary (19, 31) but also CRPC xenograft tumors in nude mice (31).

Stat5a/b regulation of prostate cancer cell viability involves both AR-dependent and AR-independent mechanisms (18, 19, 21, 25, 29). We have shown previously that Stat5a/b and AR functionally synergize in prostate cancer to enhance nuclear localization and transcriptional activity of both proteins (29). At the same time, it is well established that AR activity in prostate cancer is regulated not only at the transcriptional level but also by translational and posttranslational mechanisms (32). Recently, Stat5a/b was proposed to be involved in upregulation of AR levels in prostate cancer cells when AR is liganded by androgens (25). Here, we sought to investigate mechanistically if Stat5a/b regulates AR when liganded by antiandrogens, or when AR is unliganded during androgen deprivation of prostate cancer cells. In this work, we show for the first time that Stat5a/b protects both unliganded and antiandrogen-ligated AR from proteasomal degradation in prostate cancer. Antiandrogens induce proteasomal degradation of AR, which can be accelerated by disruption of Stat5a/b activity. Maximal loss of AR protein and inhibition of AR-driven prostate cancer cell growth is achieved through a combination of antiandrogen treatment and Stat5a/b inhibition. Our results highlight a novel means of exploiting AR proteasomal degradation by targeting of Stat5a/b to potentially improve efficacy of antiandrogens in prostate cancer.

Materials and Methods

Cell lines and reagents

Human prostate cancer cell lines LNCaP, CWR22Rv1, and PC-3 (ATCC) were cultured in RPMI-1640 (Mediatech, Inc.) containing 10% fetal bovine serum (FBS; Quality Biological) and penicillin/streptomycin (Mediatech, Inc.; 50 IU/mL and 50 μg/mL, respectively), LAPC-4 cell line (Provided by Dr. Charles Sawyers in 2012, Sloan-Kettering Memorial Cancer Center, NY) was maintained in IMDM (Mediatech, Inc.) supplemented with 1% penicillin/streptomycin and 1-glutamine and 10% FBS. LNCaP and LAPC-4 cells were cultured in the presence of 0.5 nmol/L dihydrotestosterone (DHT; Sigma-Aldrich). Human prolactin (Prl) was obtained from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA), flutamide and bicalutamide from Selleck Chemicals, MDV3100 (enzalutamide) from MedChem Express, and IST5-002 was synthesized by Fox Chase Chemical Diversity Center (Doylestown, PA). All cell lines included in this study have been authenticated on a regular basis in the users’ laboratory. The testing has been conducted by observation of characteristic cell morphology, androgen-responsiveness, and the expression of cell line–specific markers such as PSA, androgen receptor, Stat3/Stat5, and Erk1/2 protein. CWR22Rv1 cells were obtained in 2005 from Dr. Thomas Pretlow (Case Western Reserve University, Cleveland, OH) and LNCaP and DU145 cells in 2009 from ATCC.

Protein solubilization and immunoblotting

Cell pellets were solubilized in lysis buffer [10 mmol/L Tris-HCl (pH 7.6), 5 mmol/L EDTA, 50 mmol/L sodium chloride, 30 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 1 μg/mL pepstatin A, and 2 μg/mL leupeptin; refs. 19, 21, 22, 29]. Protein concentrations of cell lysates were determined by simplified Bradford method (Bio-Rad). The primary antibodies used were

Figure 1.
Activation of Stat5a/b by tyrosine phosphorylation is required for Stat5a/b–induced nuclear translocation of unliganded and antiandrogen-ligated AR in prostate cancer cells. A, I, prolactin (Prl) stimulation increases nuclear levels of WT Stat5a/b with a concurrent increase in nuclear levels of AR in PC-3 cells. Increased nuclear levels of Stat5a/b and AR are observed in the presence of (ii) phosphorylation-dead Stat5a/b(Y694F) or (iii) DN Stat5a/b. PC-3 cells were infected with AdAR, AdPrlR, and AdWTStat5a, AdDNStat5a/b or AdStat5a(Y694F; each at MOI – 4) and serum-starved (0% FBS) for 12 hours before stimulation with 10 nmol/L Prl for 30 minutes and/or 1 nmol/L DHT for 60 minutes. Cells were immunostained for AR, and, where indicated. Merged imaging indicates overlay of Stat5a/b and AR localization and transcriptional activity of both proteins (29).
anti-Stat5a/b mAb (1:1,000; BD Biosciences), anti-androgen receptor (AR) mAb (1:1,000, BioGenex Laboratories), anti-prostate-specific antigen (PSA) pAb (1:1,000; Dako), and anti-β-actin pAb (1:2,000; Sigma-Aldrich).

Protein coimmunoprecipitation
LNCaP cells were infected with adenovirus expressing PrlR (MOI, 4), serum-starved overnight in 0.5% FBS, and stimulated with or without 10 nmol/L Prl and 1 nmol/L DHT, 10 μmol/L bicatulateamide, 10 μmol/L flutamide, or 10 μmol/L MDV3100 for 1 hour. Stat5a and Stat5b were immunoprecipitated from whole-cell lysates with anti-Stat5a or anti-Stat5b (4 μl/mL; Millipore) versus IgG from normal rabbit serum (4 μl/mL; Sigma). Antibodies were captured by incubation for 60 minutes with protein A-Sepharose beads (Amersham Pharmacia Biotech). Primary antibodies used for immunoblotting were anti-AR mAb (1:1,000; Biogenex Laboratories) and anti-Stat5a/b mAb (1:1,000; BD Biosciences). The cell lysates were separated on a 4% to 12% SDS-PAGE (Life Technologies) and transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore). For immunoblotting, blocking buffer used was Tris-buffered saline and Tween 20 (TBST, 0.15 mol/L NaCl; 0.1% Tween 20; 50 mmol/L Tris, pH 8.0) with 3% bovine serum albumin (BSA). The immunoreaction was detected by horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (1:2,000; BD Biosciences).

Double immunofluorescence cytochemistry of Stat5a/b and AR
PC-3 cells were infected with AdWTStat5a, AdDStat5a(Y694F), AdWPrlR, and AdWTAR each at MOI 4, as indicated (Fig. 1A, B, and D). LNCaP cells were infected with AdPrlR at MOI 4 (Fig. 1C). The cell were serum-starved overnight before stimulation with 10 nmol/L Prl (30 minutes) and/or 1 nmol/L DHT (60 minutes). In Fig. 1D, PC-3 cells were treated with 1 nmol/L DHT, 10 μmol/L flutamide, 10 μmol/L bicatulateamide, or 10 μmol/L MDV3100 for 1 hour before stimulation with 10 nmol/L Prl (30 minutes). Cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 (Sigma), and incubated with anti-Stat5p pAb (1:200; Santa Cruz Biotechnology) and anti-AR mAb (1:200; Santa Cruz Biotechnology) followed by goat anti-rabbit fluorescein IgG (1:200; Vector Laboratories), and horse anti-mouse Texas Red IgG (1:200; Vector Laboratories), respectively. Immunofluorescence staining was detected by a Zeiss LSM 510 laser scanning microscope with an Apochromat X63/1.4 oil immersion objective (Zeiss).

Quantitative real-time polymerase chain reaction
RNA was extracted from LNCaP or LAPC-4 cells using the RNeasy Mini Kit (Qiagen) and reverse-transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase (200 U/L) overnight. Primers used were as follows: PSA (F, 5'-CATCAGGAACAAACCCGGCTGA-3' and R, 5’-AGGTTGGCTGCAGGCTGAAAT-3'), TMPRSS2 (F, 5’-CTGATGGTAAATCAGGAC-TGG-3' and R, 5’-CAGCCCATCTTCTTTCTGGTA-3'), AR (F, 5’-AACAGAATCCACTGTCGCC-3' and R, 5’-TTTACGATACAC-GTCTTTGCACC-3'), and GAPDH (F, 5’-TCAAGAGGTCGTGGAAGCAG-3' and R, 5’-CTTACTCCCTGGAGGCAATG-3').

Chromatin immunoprecipitation assay
LNCaP cells were cross-linked with 1% formaldehyde, quenched with 125 mmol/L glycine, and solubilized in lysis buffer (10 mmol/L HEPES 7.5, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 0.5 mmol/L DTT, 0.5% NP-40, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 1 μg/mL pepstatin A, and 2 μg/mL leupeptin). Nuclei were pelleted from whole-cell lysates and solubilized in nuclear lysis buffer (50 mmol/L Tris pH 8.0, 10 mmol/L EDTA, 1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 1 μg/mL pepstatin A, and 2 μg/mL leupeptin). Nuclear lysates were sonicated to shear DNA, yielding fragments of approximately 500 bp. Extracts were precleared using protein A-Sepharose beads (Amersham Pharmacia Biotech) and diluted 5-fold with RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris pH 8.0, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) before immunoprecipitation with anti-AR pAb (1.2 μg/mL, Santa Cruz Biotechnology). Immunoprecipitated complexes were washed twice with Super RIPA buffer (275 mmol/L NaCl, 50 mmol/L Tris pH 8.0, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) and diluted with 100 μL of elution buffer (0.1 mol/L NaHCO3, 1% SDS). Cross-linking was reversed by heating at 65°C overnight in 5 mol/L NaCl. DNA was extracted using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol and subjected to PCR amplification. Primers used for PSA promoter were forward 5’-TC1CCCTCCCTGCTCAGAT-3’ and reverse 5’-AACCTTCATTCCCCAGGACT-3’.

Generation of Stat5a and AR deletion constructs
Full-length Stat5a and AR were amplified by PCR and subcloned to the pCMV-3FLAG vector (Stratagene) with EcoRI and BglII sites. Stat5a deletion constructs were constructed by PCR-directed mutagenesis, with pCMV-3FLAG-Stat5a (full-length Stat5a) as used as a template. PCR primers for each Stat5a deletion construct contain different Stat5a truncated boundary sequences. PCR products were digested with EcoRI/BglII and ligated with the pCMV-3FLAG vector to form Stat5a deletion constructs and subjected to sequencing analysis for verification. Full-length AR was amplified by PCR and subcloned to pCMV-3MYC vector (Stratagene) with BamHI and Xhol sites. AR deletion constructs were then generated and verified as described for Stat5a deletion constructs.

Coimmunoprecipitation of FLAG-Stat5a and MYC-AR deletion constructs
PC-3 cells were cotransfected with plasmid pCMV-3FLAG-Stat5a deletion constructs, pCMV-3MYC-AR (full-length AR) and pPrlR (Fig. 3D) or pCMV-3MYC-AR deletion constructs, pCMV-3FLAG-Stat5a (full-length Stat5a) and pPrlR (Fig. 3E) using FuGENE6 (Promega). Cells were serum-starved and stimulated with 10 nmol/L Prl and 1 nmol/L DHT for 16 hours. The antibodies used for the coimmunoprecipitations were anti-AR pAb (200 μg/mL; Santa Cruz Biotechnology), 25 μL anti-FLAG pAb (1 μg/mL; Stratagene) or rabbit IgG polyclonal (12 mg/mL; Sigma) followed by immunoblotting with anti-FLAG mAb (1:1,000; Stratagene) or anti-MYC mAb (1:1,000; Sigma).

Generation of adenoviruses for gene delivery of wild-type, dominant-negative, and phosphorylation-dead (Y694F) Stat5a and wild-type AR
pcDNA-CMV-WT Stat5a, pcDNA-CMV-DNStat5a, pcDNA-CMV-Stat5a(Y694F), and pAR constructs were cloned to adenoviral vector using the BD Adeno-X Expression System 2 (BD Biosciences Clontech) per the manufacturer's protocol. Purified recombinant adenoviruses were linearized by Pac1 digestion.
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and transfected to QBI-293A cells (Qbioine) to produce infectious adenoviruses. AdWTStat5a, AdDNStat5, AdStat5a (Y694F), and AdWTAR viral stocks were expanded in large-scale cultures, purified using double cesium chloride gradient centrifugation, and titrated by a standard plaque assay method in QBI-293A cells, as per the manufacturer’s instructions. AdPrlR was a gift from Dr. Hallgeir Rui (Thomas Jefferson University, Philadelphia, PA).

Generation of Stat5a/b antisense oligodeoxynucleotides and transfection of prostate cancer cells

Stat5a/b ASO (5’-GGGCCCCTGGTCCATGTACGTTG-3’; shared sequence within both human Stat5a and Stat5b transcripts; bp 2153–2173 in open reading frame) were synthesized by Isis Pharmaceuticals, using a phosphorothiate backbone with 2’-O-methoxymethyl modification of five terminal nucleotides (underlined) to increase stability (ISIS 130826). As control, mismatch (MM) oligodeoxynucleotides for the same chemistry were synthesized as a mixture of all four nucleotide bases. LNCaP cells were transfected using the jetPEI reagent (Qbiogene) with Stat5a/b ASO or MM control (each at 900 pmol) for 48 hours.

shRNA constructs and lentivirus production

The RNAi consortium (TRC) pLKO.1 lentiviral vectors containing shRNA targeting AR (shAR), Stat5a (shStat5a), Stat5b (shStat5b), or scrambled control sequence (shCtrl) were purchased from Thermo Fisher Scientific. Second-generation VSVG pseudotyped high-titer lentiviruses were generated by transient cotransfection of 293FT cells, kindly provided by Dr. Hallgeir Rui (Thomas Jefferson University, Philadelphia, PA), with a three-plasmid combination as follows: one T75 cell culture flask containing 8 × 10⁶ 293FT cells was transfected with 9 μg pLKO.1 lentivector containing shRNA of interest, 10 μg pHR8.2ΔR packaging plasmid and 1 μg pCMV-VSVG envelope plasmid using Lipofectamine 2000 (Life Technologies) in Opti-MEM (Life Technologies).

Human prostate cancer xenograft studies

Castrated male athymic nude mice (Taconic), cared for according to institutional guidelines of Thomas Jefferson University, were implanted with sustained-release DHT pellets (60-day release, 1 pellet/mouse; Innovative Research of America) 3 days before inoculation of CWR22Rv1 cells. Briefly, 1.5 × 10⁶ CWR22Rv1 cells were combined with 0.2 mL of Matrigel (BD Biosciences) and implanted subcutaneously (s.c.) into flanks of nude mice (one tumor/mouse) as previously described (19, 21, 31). After tumors reached approximately 100 mm³ in size (1 week), mice were randomized into four groups (n = 10 per group) and treated daily for 10 days by intraperitoneal (i.p.) injections with 0.2 mL of IST5-002 dissolved in 0.3% hydroxypropyl cellulose (HPC; Sigma-Aldrich) at 25 mg/kg or 50 mg/kg body weight, or 0.3% HPC solution alone (vehicle), or received no treatment. Tumor sizes were measured using calipers three times per week and tumor volumes were calculated using the following formula: (3.14 × length × width × depth)/6. When tumors reached 15 to 20 mm in diameter in the control groups, mice were sacrificed and tumor tissues were harvested.

Clinical prostate cancer sample collection, subjects, and clinical information

Clinical samples, consisting of formalin-fixed, paraffin-embedded material from 132 radical prostatectomies performed as primary therapy for clinically localized prostate adenocarcinoma, were collected from the pathology archives at Turku University Central Hospital (Turku, Finland) during 1986–1997. None of the patients received adjuvant therapy before or right after surgery. Clinical postprostatectomy follow-up data and clinical information on pre- and postoperative conditions were collected retrospectively from patient files in the hospital archives and the information about the cause and date of death was obtained from the National Center for Statistics (Statistics Finland). The study protocol was approved by the ethical committee of the University of Turku (Turku,
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AR proteasomal degradation analysis

Cells were treated with vehicle (dimethylsulfoxide, DMSO; Sigma), 1 nM/L DHT, 10 μM/L flutamide, bicalutamide or MDV3100 and/or transiently transduced with lentiviral shRNA targeting Stat5a/b, where indicated (Figs. 4A and B and 5A, C, and D). Cells were treated with 10 μM/L MG132 (Calbiochem) 6 hours before harvest and immunoblotted as previously described. In Fig. 4Bi, cells were also treated with 10 μg/mL cyclohexamide (CHX; Calbiochem) 6 hours before harvest.

Cell viability assay

Cell viability was analyzed by the CellTiter 96 Aqueous Assay Kit (Promega) according to the manufacturer’s protocol.

Tissue microarray construction

Before preparation of the actual tissue microarray (TMA), all hematoxylin & eosin–stained slides from prostatectomy specimens and metastases were reevaluated to identify representative areas of benign, low-grade prostatic intraepithelial neoplasia (LGPIN), high-grade PIN (HGPIN), and carcinoma lesions. When possible, multiple cores per patient (range, 1–20; mean, 4.55) were collected. Carcinoma cores in TMs were graded only according to the predominant Gleason pattern because of the small amount of tissue in each transferred core. TMs with 55 separate cores in each block were constructed with a manual tissue arrayer (Beecher Instruments).

AR proteasomal degradation analysis

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Cell viability assay

Cell viability was analyzed by the CellTiter 96 Aqueous Assay Kit (Promega) according to the manufacturer’s protocol.

Figure 3. Active Stat5a/b upregulates expression of AR-regulated genes, increases AR occupancy of the PSA promoter, and physically interacts with antiandrogen-ligated AR in prostate cancer cells. A, a disruption of Stat5a/b activity through genetic knockdown (left) or IST5-002 treatment (right) downregulates mRNA levels of AR target genes (i) PSA and (ii) TMPRSS2, but does not affect mRNA levels of AR (iii). LNCaP cells were transduced with lentiviral shRNA targeting Stat5a/b (shStat5a/b) or scrambled control sequence (shCtrl), or treated with 25 μM of AR target genes (i) PSA and (ii) TMPRSS2, but does not affect mRNA levels of AR (iii). LNCaP cells were transduced with lentiviral shRNA targeting Stat5a/b (shStat5a/b) or scrambled control sequence (shCtrl), or treated with 25 μM of AR target genes (i) PSA and (ii) TMPRSS2, but does not affect mRNA levels of AR (iii). LNCaP cells were transduced with lentiviral shRNA targeting Stat5a/b (shStat5a/b) or scrambled control sequence (shCtrl), or treated with 25 μM of AR target genes (i) PSA and (ii) TMPRSS2, but does not affect mRNA levels of AR (iii).
domain. The cells were stimulated with 10 nmol/L human prolactin to activate Stat5a/b. As we have demonstrated previously (29), Stat5a/b activation resulted in increased levels of both Stat5a/b and AR proteins within the nuclear compartment, as detected by increased immunofluorescence of Stat5a/b and AR in the nucleus (Fig. 1A, i). In contrast, adenoviral delivery of either phosphorylation-dead Stat5a (AdStat5aY694F) or AdDNStat5 in place of wild-type Stat5, followed by prolactin stimulation, failed to induce comparable nuclear localization of Stat5a/b or AR, shown by equivalent distribution of Stat5a/b and AR immunofluorescence between cytosol and nucleus (Fig. 1A, ii and iii). Stimulation of PC-3 cells with 1 nmol/L DHT, however, induced nuclear localization of AR with concurrent increased nuclear localization of wild-type Stat5, phosphorylation-dead Stat5a(Y694F) and AdDNStat5 (Fig. 1A). These data indicate that transcriptionally active, phosphorylation-competent Stat5a/b is required for nuclear localization of unliganded AR in prostate cancer cells.

In a parallel set of experiments, serum-starved PC-3 cells were infected with adenovirus expressing wild-type Stat5a and prolactin receptor but not AR (Fig. 1B, i) or wild-type Stat5a and AR but not prolactin receptor (Fig. 1B, ii). Although direct Stat5a/b activation via prolactin stimulation still resulted in nuclear localization of Stat5a/b, in the absence of AR protein, the nuclear localization of Stat5a/b following DHT stimulation was not detectable (Fig. 1B, i). In addition, the absence of prolactin receptor to transduce signaling to Stat5a/b after prolactin stimulation abrogated not only nuclear localization of Stat5a/b, but of AR as well. However, DHT stimulation in the absence of prolactin receptor was still capable of increasing nuclear protein levels of not only AR, but also Stat5a/b (Fig. 1B, ii). A time-course analysis of LNCaP cells, which endogenously express both AR and

Figure 4. Stat5a/b genetic knockdown decreases AR protein levels, which is partially rescued by proteasome inhibition in prostate cancer cells. A, genetic knockdown of Stat5a/b decreases (i, ii) AR and (iii, iv) PSA protein levels in LNCaP and LAPC-4 cells. LNCaP and LAPC-4 cells were transduced with lentiviral shStat5a/b, shAR, or shCtrl for 72 hours, followed by immunoblotting of cell lysates with anti-AR mAb, anti-Stat5a/b mAb, anti-PSA pAb, or anti-β-actin pAb. In i, LNCaP cells were treated with 1 nmol/L DHT 24 hours before harvest and immunoblotting. In iv, LNCaP cells were transfected with ASOs targeting Stat5 or mismatched (MM) sequence as control for 72 hours. B, i and ii, Stat5a/b knockdown-mediated decrease of AR protein levels occurs through AR proteasomal degradation and iii is not attributable to increased AR protein synthesis. LNCaP cells were transduced with lentiviral shStat5a/b or shCtrl for 72 hours and treated with 10 μmol/L MG132 (proteasomal inhibitor) 6 hours before harvest, followed by immunoblotting of cell lysates with anti-AR mAb, anti-Stat5a/b mAb, anti-PSA pAb, and anti-β-actin pAb. In ii, cells were harvested at 24, 48, and 72 hours after lentiviral transduction. In iii, cells were treated with 10 μg/mL cyclohexamide (protein synthesis inhibitor) 6 or 48 hours before harvest.
Stat5a/b, confirmed that active Stat5a/b induces localization of AR in the nuclei of prostate cancer cells (Fig. 1C). Peak nuclear localization of both Stat5a/b and AR concurrently occurred at 30 minutes after DHT stimulation (Supplementary Fig. S1) and at 10 minutes after prolactin stimulation (Supplementary Fig. S2). Collectively, these data indicate that active, tyrosine-phosphorylated Stat5a/b promotes nuclear localization of unliganded AR in prostate cancer cells.

To evaluate if active Stat5 is capable of inducing nuclear localization of AR liganded by antiandrogens in prostate cancer cells, PC-3 cells expressing Stat5a/b, prolactin receptor, and AR were treated with DHT, flutamide, bicalutamide, or MDV3100 followed by prolactin stimulation and immunocytochemistry of Stat5a/b and AR. DHT stimulation resulted in increased nuclear levels of both Stat5a/b and AR in the absence of prolactin (Fig. 1D). Intriguingly, prolactin-stimulated activation of Stat5a/b induced nuclear localization of not only AR liganded by DHT, but AR liganded by flutamide, bicalutamide, or MDV3100 as well (Fig. 1D). These results suggest that active Stat5a/b can circumvent antiandrogen action in leading to increased nuclear levels of AR liganded by flutamide, bicalutamide, or MDV3100.

To investigate if high levels of nuclear Stat5a/b and AR proteins are associated in prostate cancer in vivo, we first analyzed CWR22Rv1 xenograft tumors grown in nude mice treated with a small-molecule Stat5a/b dimerization inhibitor, IIST5-002, for nuclear Stat5a/b and AR content. As expected, IIST5-002 decreased nuclear Stat5a/b/protein levels in a dose-dependent manner, as shown by Stat5a/b immunostaining of tumor sections accompanied by quantification (Fig. 2A). At the same time, AR immunostaining and quantification revealed that IIST5-002 decreased nuclear AR protein levels in a dose-dependent manner as well (Fig. 2A). Next, we analyzed a cohort of 443 clinical prostate cancer samples (Supplementary Table S1) assembled in tissue microarray for nuclear Stat5a/b and AR content by immunostaining with quantitative scoring. A positive association was demonstrated between the levels of nuclear Stat5a/b and AR proteins, with mixed effects regression indicating that high nuclear Stat5a/b protein levels were significantly associated with high AR protein levels (P < 0.0001; Fig. 2B). In summary, the reciprocal interaction of Stat5a/b and AR characterized in prostate cancer cell lines extends to human prostate cancer xenograft tumors grown in vivo and patient-derived clinical prostate cancer samples.

Active Stat5a/b enhances AR-regulated gene expression in prostate cancer cells and physically interacts with antiandrogen-liganded AR

Given that active Stat5a/b induced nuclear localization of antiandrogen-liganded AR in prostate cancer cells, we next investigated if active Stat5a/b modulates AR-regulated gene expression in prostate cancer. We disrupted Stat5a/b activity in LNCaP cells by genetic knockdown using lentiviral delivery of shRNA targeting Stat5a/b (shStat5a/b). Quantitative RT-PCR revealed that mRNA levels of PSA (P = 0.044) and transmembrane protease, serine 2 (TMPRSS2; P = 0.009), two well-known representatives of AR-regulated genes (37, 38), were decreased by 50% or more after 48 hours compared with controls (Fig. 3A, i and ii). In the next set of experiments, Stat5a/b signaling was blocked by treatment of the cells with Stat5a/b dimerization inhibitor IIST5-002 (25 μmol/L). Pharmacologic inhibition of Stat5a/b decreased both PSA (P = 0.029) and TMPRSS2 (P = 0.078) mRNA expression to the same extent as genetic knockdown of Stat5a/b (Fig. 3A, i and ii). This decrease in the expression of several AR target genes was not due to decreased AR mRNA expression, as AR mRNA levels remained unperturbed, or even slightly increased, 48 hours after Stat5a/b knockdown or IIST5-002 treatment (Fig. 3A, iii). To determine if active Stat5a/b increases occupancy of AR at promoter regions within AR-regulated genes, we selected the PSA promoter for our investigation. Serum-starved LNCaP cells with endogenous Stat5a/b and AR proteins were stimulated with 10 nmol/L prolactin and/or 1 nmol/L DHT, followed by chromatin immunoprecipitation–PCR to identify binding of AR to a sequence within the promoter of the PSA gene. Prolactin stimulation enhanced AR occupancy of the PSA promoter in the absence of androgens (Fig. 3B). These results suggest that Stat5a/b up-regulates AR signaling in prostate cancer cells at the protein level and not by regulating AR mRNA expression.

To evaluate if Stat5a/b physically interacts with AR when liganded by the antiandrogens flutamide, bicalutamide and MDV3100 in prostate cancer cells, Stat5a or Stat5b were immunoprecipitated from LNCaP cells and immunoblotted with anti-AR antibody. The results demonstrate that Stat5a/b physically interacts with AR liganded not only with DHT but also with flutamide, bicalutamide, or MDV3100 (Fig. 3C). To identify the Stat5a/b and AR domains responsible for mediating interaction of the two proteins, we generated a series of truncated FLAG-tagged Stat5a/b and MYC-tagged AR deletion constructs. Full-length MYC-AR was cotransfected into PC-3 cells along with each of the listed FLAG-Stat5a/b deletion constructs individually (Fig. 3D); full-length (FL); N-terminal-deleted (NTD); N-terminal and coiled-coil-deleted (NTCCD); C-terminus-deleted (N-ter); N-terminus-deleted (C-ter). Immunoprecipitation of MYC-AR followed by immunoblotting of FLAG-Stat5a/b revealed that Stat5a/C-ter, corresponding to a deletion of the Stat5a N-terminal and coiled-coil domains and a partial deletion of the Stat5a DNA-binding domain, was the only construct that failed to coimmunoprecipitate with MYC-AR (Fig. 3D). Analysis of the overlap of the domains represented by Stat5a/NTCCD, N-ter and C-ter suggest that the AR interaction site is localized to the N-terminal portion of the Stat5a DNA-binding domain.

In the reverse scenario, full-length FLAG-Stat5a/b was cotransfected into PC-3 cells along with each of the listed MYC-AR deletion constructs individually [Fig. 3E; 1–565 (Δ566–919); 566–919 (Δ1–565); 1–670 (Δ671–919); 1–608 (Δ609–919)]. Immunoprecipitation of FLAG-Stat5a followed by immunoblotting of MYC-AR revealed that AR–566–919, corresponding to a deletion of the AR N-terminal activation domain (Δ1–565), was the only construct that failed to coimmunoprecipitate with Stat5a (Fig. 3E). Analysis of the overlap of the domains represented by the other AR deletion constructs indicated that the Stat5a interaction site is localized, albeit broadly, to the AR N-terminus between amino acids 1 and 565. To summarize, Stat5a/b physically interacts with antiandrogen-liganded AR in prostate cancer cells, and the DNA-binding domain of Stat5 and the N-terminal domain of AR contribute to physical interaction of Stat5a/b and AR.

Stat5a/b protects both androgen- and antiandrogen-liganded AR from proteasomal degradation in prostate cancer cells

Having established functional importance of the Stat5a/b-AR interaction in prostate cancer and given that genetic knockdown of Stat5a/b did not affect AR mRNA levels, we wanted to evaluate...
the possibility that Stat5a/b regulates AR protein levels during androgen deprivation of prostate cancer cells. We first analyzed protein levels of AR in the presence or absence of androgens following disruption of Stat5a/b function in prostate cancer cells. In both LNCaP and LAPC-4 cells, genetic knockdown of Stat5a/b (shStat5a/b) resulted in a substantial loss of AR protein after 72 hours, as visualized by immunoblotting of whole-cell lysates (Fig. 4A, i and ii). Treatment with 1 nmol/L DHT reversed this trend and partially prevented downregulation of AR protein levels (Fig. 4A, i). Interestingly, Stat5a/b knockdown was almost as efficient as direct knockdown of AR (shAR) in decreasing AR protein levels (Fig. 4A, ii). PSA protein levels were also diminished following Stat5a/b knockdown by Stat5a/b antisense oligonucleotides during this timeframe, presumably due to loss of AR protein (Fig. 4A, iii and iv).

To assess if loss of AR protein after Stat5a/b knockdown was due to increased flux of AR through the proteasome, we initiated Stat5a/b knockdown followed by treatment with 10 μmol/L proteasome inhibitor MG132. Proteasomal blockade partially rescued Stat5a/b knockdown-induced loss of AR, restoring AR protein levels in prostate cancer cells (Fig. 4B, i). AR loss, and subsequent AR rescue through proteasome inhibition, was Stat5a/b-dependent, requiring prior Stat5a/b knockdown, which generally occurred 48 to 72 hours after transduction of cells with lentiviral shStat5a/b (Fig. 4B, ii). AR rescue could not be attributed to generation of new AR protein, as rescue of AR protein levels occurred to a comparable extent in the presence of both MG132 and protein synthesis inhibitor cyclohexamide (Fig. 4B, iii).

Because active Stat5a/b induced nuclear localization of antiandrogen-liganded AR and physical interaction between Stat5a/b and antiandrogen-liganded AR, we next investigated if active Stat5a/b decreases proteasomal degradation of AR liganded by antiandrogens in prostate cancer cells. To evaluate the basal levels of AR in the presence of androgens versus antiandrogens in prostate cancer cells, AR was immunoblotted from LNCaP and LAPC-4 cells treated with DHT, flutamide, bicalutamide, or MDV3100 for 2, 3, 4, or 5 days. Unexpectedly, the data showed that antiandrogens decreased the mRNA levels of AR. AR mRNA levels remained unaffected or were slightly increased by antiandrogen treatment during the same timeframe (LNCaP; bicalutamide, flutamide P = 0.0004, MDV3100 P = 0.037; LAPC-4; bicalutamide, flutamide P = 0.1213, MDV3100 P = 0.011; Fig. 5B). To investigate the mechanism of antiandrogen regulation of AR protein levels in prostate cancer cells, we treated LNCaP and LAPC-4 cells with MG132 in the presence of absence of flutamide, bicalutamide, or MDV3100. As with Stat5a/b knockdown, the data suggest that antiandrogen treatment induced AR loss through increased proteasomal degradation in prostate cancer cells, as AR protein levels could be rescued by treatment with MG132 (Fig. 5C).

If Stat5a/b protects AR liganded by antiandrogens from proteasomal degradation in prostate cancer cells, targeting Stat5a/b in the presence of antiandrogen-ligated AR could potentially provide a strategy to accelerate proteasomal degradation of AR. To test this concept, Stat5a/b was genetically knocked down by shRNA followed by treatment with DHT, flutamide, bicalutamide, or MDV3100 (Fig. 5D). In all instances, antiandrogen-ligated AR or AR in the absence of Stat5a/b was subjected to enhanced degradation. Indeed, the combination of Stat5a/b knockdown and antiandrogen treatment reduced AR protein levels to a greater extent than either intervention alone in LNCaP cells (Fig. 5D, i). Enhanced proteasomal degradation of AR through combined Stat5a/b knockdown and antiandrogen treatment was partially rescued by treatment with MG132, as previously seen for each intervention individually (Fig. 5D, ii). In conclusion, the data presented here indicate that Stat5a/b disruption potentiates AR proteasomal degradation induced by antiandrogens.

Combined inhibition of Stat5a/b and AR suppresses growth of prostate cancer cells to a greater extent than AR inhibition alone

To determine if pharmacologic Stat5a/b inhibition potentiates the growth-inhibitory effects of antiandrogens, we evaluated the efficacy of combination therapy of antiandrogen MDV3100 and Stat5a/b dimerization inhibitor IST5-002 to MDV3100 alone in LNCaP cells. First, we determined sensitivity of LNCaP growth to MDV3100 (IC_{50} = 90 μmol/L; Fig. 6A) or IST5-002 (IC_{50} = 25 μmol/L; Fig. 6B) as single agents. Next, LNCaP cells were treated with increasing doses of MDV3100 either alone or in combination with 25 μmol/L LST5-002 (IC_{50} value). At every dose of MDV3100 tested, a combination of MDV3100 and IST5-002 inhibited growth of LNCaP cells to a greater extent than MDV3100 alone (P < 0.0001; Fig. 6C). Remarkably, the addition of IST5-002 decreased the IC_{50} value of MDV3100 from 90 μmol/L to approximately 5 μmol/L in LNCaP cells, indicating substantial potentiation of antiandrogen action by Stat5a/b inhibition (P < 0.0001; Fig. 6A, iii). Collectively, these results suggest that efficacy of antiandrogens can be improved by simultaneous blockade of Stat5a/b signaling.

Discussion

In the present work, we demonstrate for the first time that Stat5a/b protects antiandrogen-ligated AR from proteasomal degradation in prostate cancer cells. Our results show that active
One of the key results of this work is the finding that Stat5a/b increased nuclear levels of antiandrogen-ligated AR and that transcriptionally active, phosphorylation-competent Stat5 is required for nuclear sequestration of AR. This finding is important because it suggests that pharmacologic inhibitors of the Stat5a/b signaling pathway have the potential to downregulate nuclear AR content in prostate cancer cells, resulting in decreased levels of AR available within the nucleus. We further demonstrated that active Stat5a/b and antiandrogen-ligated AR physically interact in prostate cancer cells, forming a Stat5a/b–AR complex detectable by coimmunoprecipitation (29). At present, it is unclear if the formation of a Stat5a/b–AR complex prevents recruitment of E3 ubiquitin ligases targeting AR, impedes ubiquitination of AR or interferes with some other event before AR being directed to the proteasome. AR ubiquitination depends on AR phosphorylation, with phosphorylated serine or tyrosine residues serving as recognition motifs for E3 ligases (phosphodegrons; refs 43, 46, 47). Phospho-S515 and -Y534 of AR represent the phosphodegrons. Analogous regulation of transcriptional activity and nucleo-cytoplasmic translocation, transcriptional activity, and coactivator recruitment are well-documented (16, 17, 44), the effects of antiandrogens on AR nuclear translocation, transcriptional activity, and coactivator recruitment are well-documented (16, 17, 44), the effects of antiandrogens on AR protein half-life is less clearly understood (45). Our data provide critical evidence supporting the notion that both first- and second-generation antiandrogens decrease AR protein levels in prostate cancer cells, while not reducing AR mRNA levels. Importantly, our results further show that active Stat5a/b potentiates AR signaling in the presence of antiandrogens by impeding the proteasomal degradation of antiandrogen-ligated AR in prostate cancer cells. Because AR-regulated gene expression is thought to be maintained during progression to CRPC and development of antiandrogen resistance, it is possible that Stat5a/b mediates sustained activity of AR in the presence of antiandrogens. Stat5a/b activation also induced other downstream molecular events involved in AR signaling, resulting in increased AR occupancy of the PSA promoter and expression of AR-regulated genes PSA and TMPRSS2. Future work should use whole-genome approaches to determine if Stat5a/b activation induces upregulation of the entire AR transcriptome. Whereas Stat5a/b disruption or antiandrogen treatment each individually increased flux of AR protein through the proteasome, a combination of these two therapeutic strategies induced maximal downregulation of AR protein levels through proteasomal degradation in prostate cancer cells.

We show that Stat5a/b increased nuclear levels of antiandrogen-ligated AR and that transcriptionally active, phosphorylation-competent Stat5 is required for nuclear sequestration of AR. This finding is important because it suggests that pharmacologic inhibitors of the Stat5a/b signaling pathway have the potential to downregulate nuclear AR content in prostate cancer cells, resulting in decreased levels of AR available within the nucleus. We further demonstrated that active Stat5a/b and antiandrogen-ligated AR physically interact in prostate cancer cells, forming a Stat5a/b–AR complex detectable by coimmunoprecipitation (29). At present, it is unclear if the formation of a Stat5a/b–AR complex prevents recruitment of E3 ubiquitin ligases targeting AR, impedes ubiquitination of AR or interferes with some other event before AR being directed to the proteasome. AR ubiquitination depends on AR phosphorylation, with phosphorylated serine or tyrosine residues serving as recognition motifs for E3 ligases (phosphodegrons; refs 43, 46, 47). Phospho-S515 and -Y534 of AR represent the phosphodegrons. Analogous regulation of transcriptional activity and nuclear localization has been described for BRC1 and BRC2 via molecular masking of nuclear export signals by BARD in breast cancer (48, 49). It is also possible that Stat5a/b-induced nuclear sequestration of AR in prostate cancer cells is caused by active Stat5a/b exclusively protecting nuclear-localized AR from proteasomal degradation.

Increased Stat5a/b expression may promote transition of androgen-sensitive prostate cancer to CRPC by undermining...
efficacy of antiandrogen therapy. Active Stat5a/b expression is increased in 95% of clinical CRPCs (29), the Stat5a/b gene locus is amplified in 29% of distant CRPC metastases (30), and in this work, we demonstrate a significant association between high levels of nuclear Stat5a/b and AR in patient-derived clinical prostate cancers. Importantly, we show that inhibition of Stat5a/b sensitized prostate cancer cells to treatment with the second-generation antiandrogen MDV3100, providing further evidence in support of combination therapy for prostate cancer using Stat5a/b inhibitors and antiandrogens. Collectively, these findings support the concept that upregulation of Stat5a/b signaling promotes survival of androgen-deprived prostate cancer cells. In summary, the work presented here proposes a novel means of exploiting AR proteasomal degradation and enhancing efficacy of antiandrogens through targeting of Stat5a/b. Following further clinical development, pharmacologic Stat5a/b inhibitors have the potential to be deployed alongside existing antiandrogen therapies for more effective treatment of advanced prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Inhibition of Stat5a/b Enhances Proteasomal Degradation of Androgen Receptor Liganded by Antiandrogens in Prostate Cancer

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