Human Cancer Biology

14-3-3η Amplifies Androgen Receptor Actions in Prostate Cancer

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Abstract Purpose: Androgen receptor abundance and androgen receptor–regulated gene expression in castration-recurrent prostate cancer are indicative of androgen receptor activation in the absence of testicular androgen. Androgen receptor transactivation of target genes in castration-recurrent prostate cancer occurs in part through mitogen signaling that amplifies the actions of androgen receptor and its coregulators. Herein we report on the role of 14-3-3η in androgen receptor action.

Experimental Design and Results: Androgen receptor and 14-3-3η colocalized in COS cell nuclei with and without androgen, and 14-3-3η promoted androgen receptor nuclear localization in the absence of androgen. 14-3-3η interacted with androgen receptor in cell-free binding and communoprecipitation assays. In the recurrent human prostate cancer cell line, CWR-R1, native endogenous androgen receptor transcriptional activation was stimulated by 14-3-3η at low dihydrotestosterone concentrations and was increased by epidermal growth factor. Moreover, the dihydrotestosterone- and epidermal growth factor–dependent increase in androgen receptor transactivation was inhibited by a dominant negative 14-3-3η. In the CWR22 prostate cancer xenograft model, 14-3-3η expression was increased by androgen, suggesting a feed-forward mechanism that potentiates both 14-3-3η and androgen receptor actions. 14-3-3η mRNA and protein decreased following castration of tumor-bearing mice and increased in tumors of castrate mice after treatment with testosterone. CWR22 tumors that recurred 5 months after castration contained 14-3-3η levels similar to the androgen-stimulated tumors removed before castration. In a human prostate tissue microarray of clinical specimens, 14-3-3η localized with androgen receptor in nuclei, and the similar amounts expressed in castration-recurrent prostate cancer, androgen-stimulated prostate cancer, and benign prostatic hyperplasia were consistent with androgen receptor activation in recurrent prostate cancer.


The androgenic steroid hormones, testosterone and its more active metabolite dihydrotestosterone, maintain and stimulate the growth of prostate cancer cells. Removal of the testicular source of circulating androgens by medical or surgical castration causes regression of androgen-dependent prostate cancer (1). However, androgen deprivation therapy is only palliative because prostate cancer recurs (referred to as castration-recurrent prostate cancer) and almost always causes death.

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The expression of androgen receptor (2–6) and androgen-regulated genes (7, 8) in castration-recurrent cancer implicates androgen receptor signaling in recurrent growth despite low levels of circulating androgen. Potential mechanisms include androgen receptor gene amplification (9), gain of function mutations in the androgen receptor gene resulting in promiscuous ligand binding (refs. 6, 10, see also www.androgendb.mcgill.ca), coactivator overexpression (11), intracellular production of testosterone and dihydrotestosterone (12, 13), and enhanced activation of androgen receptor through mitogen signaling (14–17).

The 14-3-3 family of homodimeric or heterodimeric α-helical proteins interact with a variety of signaling proteins including kinases, phosphatases, transcription factors, and nonkinase receptors (18). 14-3-3 proteins were the first to be identified as phosphoserine/threonine motif binding proteins. Three recognition motifs for 14-3-3 have been identified, but 14-3-3 target proteins do not always contain sequences that conform to these motifs or require phosphorylation for interaction with 14-3-3 (18, 19). Possible modes of action of 14-3-3 on target proteins include directed conformational change, modification of nuclear/cytoplasmic localization, physical occlusion of sequence-specific interactive regions, and scaffolding (20, 21). 14-3-3 binding often leads to client protein stabilization or altered subcellular localization that in many cases alters protein function. Some 14-3-3 isoforms have been directly associated with tumorigenesis and may serve as targets for cancer therapy (22).

CWR22 is an androgen-dependent human prostate cancer xenograft model propagated in male nude mice (23). After castration of the tumor-bearing male host, prostate-specific antigen protein and mRNA decrease rapidly and CWR22 xenograft tumors regress in size (7, 24) but within 5 months undergo recurrent growth in the absence of testicular androgens, a progression pattern that resembles the recurrence of prostate cancer in humans but in a shorter timeframe (23). Castration-recurrent CWR22 tumors remain androgen responsive, and an androgen-sensitive cell line, CWR-R1 (25), was developed from a recurrent CWR22 xenograft tumor.

Herein we show that 14-3-3η is a human androgen receptor-binding protein in CWR-R1 cells (25) and that 14-3-3ζ binding to androgen receptor is associated with increased androgen receptor transcriptional activity that is enhanced by epidermal growth factor (EGF) and dihydrotestosterone. We found that the expression of 14-3-3η was androgen dependent in the androgen-dependent CWR22 xenograft tumor and decreased after castration but reappeared in the castration-recurrent CWR22 tumor despite the absence of circulating testicular androgen. In addition, clinical samples of castration-recurrent cancer revealed amounts of 14-3-3ζ similar to the amounts expressed in androgen-stimulated benign prostate and androgen-stimulated prostate cancer. The results indicate that androgen receptor regulates the expression of 14-3-3η and that androgen receptor regulation is active in castration-recurrent prostate cancer. 14-3-3ζ localizes with androgen receptor in nuclei and provides a link between EGF signaling and androgen receptor transcriptional activation in prostate cancer.

### Materials and Methods

#### Plasmid construction.

Full-length 14-3-3η (18) was excised from the vector pCR-Script Amp 14-3-3ζ using EcoRI and SacI. The excised DNA was blunt-ended with Klenow enzyme (Life Technologies, Inc.) and ligated into the blunt-ended BamHI site of pSG5 (Stratagene). Sense and antisense constructs were verified by automatic sequencing using a Perkin-Elmer Corp. Model 377 DNA sequencer. A dominant negative double arginine mutant of human 14-3-3ζ cDNA (pcDNA3.1- Myc-14-3-3ζ-RS6, 60A) was obtained from Andrey S. Shaw, Washington University, St. Louis, MO.

#### Solid-phase binding assays.

Purified full-length human hexahistidine-tagged 14-3-3ζ or 14-3-3ζ was incubated with Ni2+-charged Sepharose 6B beads (Novagen) for 1 h at 4°C. Radiolabeled androgen receptor was generated using the TNT in vitro transcription-translation system (Promega). The full-length DNA template pSG5-AR was incubated with TNT rabbit reticulocyte lysates in the presence of 35S-methionine. For the binding assays (26), the immobilized 14-3-3ζ or 14-3-3ζ protein (1 μg each) was mixed with 35S-labeled androgen receptor in Nonidet P-40 buffer [1% Nonidet P-40, 137 mmol/L NaCl, 1 mmol/L MgCl2, and 40 mmol/L Tris-HCl (pH 8.0)] for 1 h at 4°C with rotation. 14-3-3ζ or 14-3-3ζ complexes were washed three times with Nonidet P-40 buffer and three times with radioimmunoprecipitation assay wash buffer (1% Nonidet P-40, 137 mmol/L NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 20 mmol/L Tris-HCl (pH 8.0)), and boiled 5 min in 2× SDS sample buffer before resolution by SDS-PAGE (12%). Cells were dried and autoradiography was done with BioMax film (Eastman Kodak Co.) at -80°C. Image analysis was carried out using the Personal Densitometer SI (Molecular Dynamics).

#### Immunoprecipitation and immunoblot analysis.

Protein lysates were prepared from COS cells grown on 10-cm culture dishes and cotransfected with sense or antisense 14-3-3ζ and human androgen receptor expression vectors as described (27). COS cells were derived from CV1 cells by transformation with replication origin defective SV40 virus that codes for large T antigen. Thus, expression vectors containing the SV40 origin of replication, when transiently transfected into COS cells, are replicated to multiple copies/cell. COS cell lysates were preclarured with mouse IgG and protein A-agarose. To examine androgen receptor/14-3-3ζ complex formation in COS cell and CWR-R1 cell lysates, anti-14-3-3ζ antibody that recognizes η and γ isoforms (Santa Cruz Biotechnology Inc.; sc-731) or anti-actin antibody (Research Diagnostic Inc.)

### Translational Relevance

We show that 14-3-3ζ is an androgen receptor coactivator in postcastration recurrent prostate cancer (CR-CaP) through its direct interaction with androgen receptor and by augmenting mitogen effects on androgen receptor transcriptional activity. The use of androgen receptor siRNA technology in the experimental setting as well as in recent phase I clinical trials with a CYP17 inhibitor of androgen production provides strong evidence that androgen receptor action is central to the growth of CR-CaP. Our studies identify a new potential target to inhibit CR-CaP growth. We show that 14-3-3ζ causes androgen receptor to be nuclear in the absence of androgen, whereas without 14-3-3ζ androgen receptor is cytoplasmic. We also show that androgen receptor signaling increases the production of 14-3-3ζ through the synthesis of new mRNA and that 14-3-3ζ levels remain elevated together with androgen receptor in CR-CaP. Studies described in this report provide a potential new target for combination therapy to block the androgen receptor–driven growth of CR-CaP.
were incubated with rotation for 1 h followed by the addition of protein A-agarose with constant rotation at 4°C overnight. Immunoblot analysis was done using androgen receptor monoclonal antibody (Biogenex) at 1:5,000 dilution and secondary antibody (horseradish peroxidase–conjugated anti-mouse, Promega Corporation) at 1:10,000 dilution. Antigen-antibody complexes were detected using enhanced chemiluminescence (DuPont, NEN Research Products).

Prostate cancer specimens were pulverized in liquid nitrogen and mixed with 0.5 mL of radioimmunoprecipitation assay buffer containing Complete protease inhibitors (Roche; ref. 11). Tissue was homogenized for 30 s on ice, incubated for 45 min on ice, and centrifuged at 10,000× g for 20 min twice at 4°C to remove nuclei and insoluble material. Proteins were separated by electrophoresis on 10% acrylamide gels, electroblotted, and immunoblot analysis was done as described above using 14-3-3ζ antibody specific for the ζ isoform (Santa Cruz sc-12787).

**Transient cotransfection assays.** Cotransfection assays using monkey kidney epithelial CV1 cells were done as described (10) with minor modifications. We have used the CV1 cell line as a reference standard for cotransfection assays with different cell lines. In brief, CV1 cells were maintained at 37°C under 5% CO2 in Dulbecco’s Modified Eagle’s Medium–High glucose (DMEM-H) medium containing 2 mmol/L L-glutamine, 10% BCS, 20 mmol/L HEPES, 100 units/mL penicillin, and 100 μg/mL streptomycin. On the day before transfection, 4.5 × 106 cells per 6-cm culture dish were grown in the same medium for about 20 h until 70% confluent. Probasin-luciferase vector (0.25 μg), 0.25 μg human androgen receptor expression vector (pSG5-hAR), and 0.5 μg of pSG5-14-3-3ζ-expression vector, or pSG5-14-3-3ζ-antisense vector per 6-cm culture dish were cotransfected using the Effectene method. After incubation for 24 h the medium was changed to DMEM-H without phenol red and pCMV-AR was transfected with the presence or absence of steroid, and the cells were incubated for 48 h.

CWR-R1 cells were maintained in prostate growth medium [DMEM-H (Gibco/Invitrogen) containing 0.1 μg/mL linoleic acid, 2 mmol/L L-glutamine, 1.2 μg/mL nicotinamide, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium, 100 units/mL penicillin, 100 μg/mL streptomycin, 20 mmol/L HEPES (pH 7.2), 2% fetal bovine serum, and 10 ng/mL EGF (mouse, BD Biosciences)]. Transient transfections were done as described (27) with minor modifications. CWR-R1 cells were plated on the day before at 106 cells per 6-cm culture dish in prostate growth medium and grown for about 20 h to approximately 70% confluence (27). Cells were cotransfected with the prostate specific antigen PSA-luciferase (referred to previously as PSE-Luc) reporter (0.5 μg) and 250 ng pSG5-14-3-3ζ-sense or pSG5-14-3-3ζ-antisense vector per 6-cm culture dish were cotransfected using the Effectene (Qiagen) method. After incubation for 24 h the medium was changed to DMEM-H without phenol red and pCMV-AR was transfected with the presence or absence of steroid, and the cells were incubated for 48 h.

CWR-R1 cells were harvested in lysis buffer (Promega), and luciferase activity was measured using a luminometer. Luciferase activity in representative assays was expressed as mean and SD of optical units from three replicates in the presence of hormone compared with replicates in the absence of hormone.

**Prostate tissue specimens.** All prostate specimens were acquired in compliance with the guidelines of the University of North Carolina at Chapel Hill Lineberger Comprehensive Cancer Center Clinical Trial Protocol Review Committee of the Institutional Review Board under the Federal Health Insurance Portability Accountability Act, and Protected Health Information Regulations. CWR22 human prostate cancer xenografts were propagated and procured as described (2, 23, 27) in compliance with approved protocols and regulations of the University of North Carolina School of Medicine at Chapel Hill Institutional Animal Care and Use Committee. Immunoassays were performed as described.

**Immunostaining of tissue sections.** Formalin-fixed, paraffin-embedded xenograft tumors were sectioned and stained using standard immunohistochemical techniques. The method for Fig. 4B1 to 4 was as follows: After being deparaffinized and rehydrated, tissue sections (8 μm) were incubated in epitope-retrieval buffer (AntigenDecloaker, pH 6.0; Biocare Medical) in a decloaking chamber (Biocare Medical) at 120°C for 5 min. Sections were pretreated with 3% hydrogen peroxide, 5% normal horse serum and avidin–biotin blocking reagents (Vector Laboratories). Rabbit polyclonal anti-14-3-3ζ antibody (Santa Cruz Biotechnology; sc-731) was used at 2 μg/mL (1:100). After incubation with biotinylated goat anti-rabbit IgG, immunostaining was amplified using Vector Laboratories Elite ABC kit and the immunoperoxidase complex was visualized using diaminobenzidine. Counterstaining was done with hematoxylin. The method for Fig. 4C1 to 6 was as follows: Tissue sections (8 μm) were incubated in 83% methanol and 5% H2O2 at room temperature for 30 min to reduce endogenous peroxidase activity, followed by exposure to 0.01 mol/L sodium citrate (pH 6.0) for 15 min in a microwave oven at high setting. For 14-3-3ζ detection using goat polyclonal anti-14-3-3ζ antibody (Santa Cruz Biotechnology), sections were blocked with 1.5% normal rabbit serum and incubated overnight at 4°C in a humidified chamber with 14-3-3ζ antibody at 0.4 μg/mL (1:500), washed with PBS, and blocked again with 1.5% normal rabbit serum. After incubation for 30 min with biotinylated rabbit antigen IgG, immunostaining was amplified using the Vector Laboratories Elite ABC Kit and the immunoperoxidase complex was visualized using diaminobenzidine. Sections were exposed to osmium vapors and counterstained with 0.05% toluidine blue in 30% ethanol, dehydrated, cleared in xylene, and mounted with Permoun (Fisher). For androgen receptor immunostaining using rabbit polyclonal anti-AR-PG21 (Upstate USA, Inc.), sections were blocked with 2% normal goat serum and incubated overnight at 4°C in a humidified chamber with AR-PG21 antibody at 0.75 μg/mL (1:200). Sections were washed with PBS and blocked again with 2% normal goat serum. Following incubation with biotinylated goat anti-rabbit IgG, immunostained AR-PG21 was amplified using Vector Laboratories Standard ABC kit. Sections were treated as above from the diaminobenzidine step to the end of the staining protocol. Photographs (+40) were taken using a SPOT-4 Megapixel Digital Color Camera System (Diagnostic Instruments, Inc.) attached to a Nikon ECLIPSE E600 microscope and prepared using SPOT image processing software.

Tissue microarray construction, immunostaining, and image acquisition were performed as described (5, 28, 29) using 14-3-3ζ-specific antibody sc-17287, lot #K1803. Tissue microarray sections were visually scored by three individuals without knowledge of tumor or patient status. Epithelial cell 14-3-3ζ nuclear and cytoplasmic immunostaining was assessed using a scale ranging from 0 (no expression) to 3 (strong expression) in each of 100 cells yielding a visual score ranging from 0 to 300.

**Immunofluorescence microscopy.** Assays were done using COS cells as described (30). Cells were grown in DMEM-H (Gibco/Invitrogen) containing 2 mmol/L L-glutamine, 20 mmol/L HEPES buffer (pH 7.2), 100 units/mL penicillin, 100 μg/mL streptomycin, and 5% BCS, and 10,000 cells/well were seeded on coverslips (Microscope coverglass, Fisher Scientific) in 12-well plates and cultured in the same medium. The following day, DEAE-dextran followed by glyceral shock was used to transfect 1 μg each of pCMV-AR, pSG FLAG-14-3-3ζ, pSG FLAG-CRIF1, or pSG FLAG alone and in the combinations shown in Fig. 1D. Cells were washed two times with TBS and incubated in DMEM-H with or without 10 mmol/L dihydrotestosterone and in the absence of serum or phenol red. Media were changed the following day and incubations continued for 24 h. Incubations with primary antibodies (anti-androgen receptor 1:2,500, and/or anti-FLAG 1:1,000) were for 1 h at room temperature. After washing three times with PBS, cells were incubated with secondary fluorescent antibodies at 1:50 dilution for 30 min at room temperature and images obtained using a Zeiss LSM 210 confocal microscope. Rabbit anti-androgen receptor ab3510 was obtained from Abcam; mouse anti-FLAG antibodies from Sigma. FITC-conjugated, AffiniPure Donkey Anti-rabbit IgG and Rhodamine (TRITC)-conjugated AffiniPure Donkey Anti-mouse IgG were from Jackson ImmunoResearch. Comparative quantitative real-time PCR analysis. Genomic DNA-free total RNA was isolated from archived CWR22 tumors (n = 2) from mice that were intact, 6 d and 12 d after castration, and recurrent
tumors from mice 150 d after castration. TRizol reagent (Gibco) was used according to the manufacturer’s protocol. Total RNA integrity was determined using the Agilent 2100 Bioanalyzer (Agilent). 14-3-3-ζ mRNA levels were assayed using β-glucuronidase as calibrator in comparative (Ct) method real-time polymerase chain reaction (PCR). Real-time PCR was done in a one-step reaction using 50 ng total RNA and 1 unit of reverse transcriptase (Roche) in a total volume of 30 μL (48°C, 30 min; 95°C, 10 min; 40 cycles at 95°C, 15 s; 63°C, 1 min) using the Applied Biosystems RT-PCR 7500 System. Specific primers for amplifying 14-3-3-ζ and β-glucuronidase were based on human GenBank sequences. Forward and reverse primer sequences were 5′-tgctccaggcagttcttcgttcc; β-glucuronidase, 5′-tgctccaggcagttcttcgttcc; 14-3-3-ζ, 5′-accattcttttcgtttccatca; and 5′-tagcttccaccctactgaggattggca and 5′-tagcttccaccctactgaggattggca. 

Statistical analyses. Mean nuclear and cytoplasmic 14-3-3-ζ immunostaining visual scores from human and CWR22 tissue microarrays, mean 14-3-3-ζ mRNA levels in CWR22 tumors, and mean densitometry values for solid phase binding assays were calculated and statistical analysis was done using Statgraphics Plus 4.1 (Manugistics, Inc.). The Mann-Whitney comparison test and Student’s t-test were used and differences were considered significant at P < 0.05.

Results

14-3-3ζ interacts with androgen receptor. His-tag pulldown experiments were done to test for a direct interaction between androgen receptor and 14-3-3ζ protein. Binding of in vitro translated [35S]labeled androgen receptor to His-tag 14-3-3ζ was positive in the presence of 1 μmol/L dihydrotestosterone compared with the Ni-Sepharose control (P = 0.013). There was no difference between control and the minus dihydrotestosterone (Fig. 1A). These results are in agreement with an earlier report by Zilliacci et al. (31), which showed that androgen receptor bound GST-14-3-3ζ in the presence of 1 μmol/L testosterone. In addition, we found that under these conditions androgen receptor did not bind the full-length 14-3-3ζ isoform, either in the presence or absence of dihydrotestosterone, suggesting a certain isoform selectivity for androgen receptor regulation. Whether androgen receptor favors the 14-3-3ζ isoform within the 14-3-3 family of proteins remains to be established.

Coimmunoprecipitation studies were carried out to test the interaction between androgen receptor and 14-3-3ζ. COS cells were cotransfected with vectors that express recombinant human full-length wild-type androgen receptor and either sense or antisense 14-3-3ζ expression vector DNA. Lysate proteins were immunoprecipitated with anti-14-3-3ζ antibody (Santa Cruz sc-731 that recognizes η and γ isoforms) and immunoblotted with androgen receptor antibody. Androgen receptor coimmunoprecipitated with 14-3-3ζ in lysates from COS cells cotransfected with sense 14-3-3ζ but not with antisense 14-3-3ζ (Fig. 1B). The amount of androgen receptor complexed with 14-3-3ζ was greater in the presence of dihydrotestosterone whereas the expression levels (input) of androgen receptor and 14-3-3ζ were similar (Fig. 1B). The androgen receptor/14-3-3ζ association seemed specific in that immunoprecipitates with anti-actin instead of anti-14-3-3ζ antibody did not contain androgen receptor. Although the interaction between recombinant wild-type androgen receptor and 14-3-3ζ was stronger in the presence of dihydrotestosterone (Fig. 1B), complex formation in the absence of dihydrotestosterone suggested the interaction was not completely androgen dependent.

When recombinant 14-3-3ζ and androgen receptor were expressed separately in COS cells, androgen receptor was mainly cytoplasmic without dihydrotestosterone and nuclear with dihydrotestosterone, whereas 14-3-3ζ was both nuclear and
cytoplasmic without or with dihydrotestosterone (Fig. 1C, top). Coexpression of 14-3-3\(\eta\) and androgen receptor in the absence of dihydrotestosterone resulted in major nuclear localization of androgen receptor and colocalization with 14-3-3\(\eta\) in nuclei (Fig. 1C, bottom). In contrast, an androgen receptor corepressor, CR6 interacting factor-1 (CRIF), localized in nuclei both in the presence and the absence of dihydrotestosterone (Fig. 1C, top) and when coexpressed with androgen receptor in the absence of dihydrotestosterone (Fig. 1C, bottom) CRIF did not alter the subcellular partitioning of androgen receptor. In the presence of dihydrotestosterone, the strong nuclear localization of androgen receptor was not altered by coexpression of 14-3-3\(\eta\) or CRIF.

14-3-3\(\eta\) enhances androgen-dependent androgen receptor transactivation. To examine the effect of 14-3-3\(\eta\) on the transcriptional function of androgen receptor, transient cotransfection assays were done in CV-1 cells. In cells transfected with androgen receptor together with a probasin-luciferase reporter gene and the sense 14-3-3\(\eta\) vector, 1 nmol/L dihydrotestosterone increased luciferase activity 3- to 4-fold greater than in control cells transfected with the antisense 14-3-3\(\eta\) (Fig. 2). In the absence of dihydrotestosterone, basal luciferase activity in cells transfected with sense 14-3-3\(\eta\) was only slightly higher than in cells transfected with antisense 14-3-3\(\eta\). In these experiments, the antisense 14-3-3\(\eta\) vector DNA was used to balance the sense 14-3-3\(\eta\) vector DNA. The results indicate that 14-3-3\(\eta\) amplifies dihydrotestosterone-induced androgen receptor transactivation in CV1 cells.

To investigate the ligand specificity of 14-3-3\(\eta\) stimulation of androgen receptor transcriptional activation, dihydrotestosterone was compared with progesterone, estradiol, and the antiandrogen hydroxyflutamide. In CV1 cell cotransfection assays using the probasin-luciferase reporter gene with dihydrotestosterone, luciferase activity with sense 14-3-3\(\eta\) was greater than with antisense 14-3-3\(\eta\). In contrast, there was little or no increase in luciferase with progesterone, estradiol, or hydroxyflutamide. Similar results were seen at more physiologic ligand concentrations of 0.1 nmol/L (not shown), suggesting that at the tissue steroid levels found in benign prostatic hyperplasia (BPH) and...
prostate cancer (12) it is androgen-induced androgen receptor transactivation that is amplified by 14-3-3\(\eta\).

14-3-3\(\eta\) enhances EGF- and dihydrotestosterone-induced androgen receptor transactivation in a castration-recurrent prostate cancer cell line. We found in earlier studies that EGF increases dihydrotestosterone-dependent androgen receptor transactivation in CWR-R1 cells (27). The EGF effect on androgen receptor action involved phosphorylation and increased coactivation by TIF2/GRIP1. Because 14-3-3 has a propensity for binding to phosphorylated proteins, we reasoned it might have a role in EGF amplification of androgen receptor transactivation. With this in mind, the effect of 14-3-3\(\eta\) on EGF and dihydrotestosterone-induced androgen receptor transactivation was determined in the CWR-R1 cell line. In the presence of the 14-3-3\(\eta\) sense vector, 0.1 nmol/L dihydrotestosterone increased androgen-dependent transcriptional activation of the PSA-luciferase reporter gene about 5-fold above the no-dihydrotestosterone control (Fig. 3A). Concurrent addition of
100 ng/mL EGF and 0.1 nmol/L dihydrotestosterone further increased androgen receptor transcriptional activity 2-fold over that of dihydrotestosterone alone. Androgen receptor transactivation in the presence of dihydrotestosterone without or with EGF was greater in the presence of the 14-3-3η sense vector, than with control 14-3-3η antisense.

Similar 14-3-3η enhancement of androgen receptor transcriptional activation was observed using the MMTV-luciferase reporter gene in CWR-R1 cells (Fig. 3B). MMTV-luciferase was a more sensitive reporter gene in these cells, and enhancement of androgen receptor transactivation in the presence of 14-3-3η was observed at lower concentrations of dihydrotestosterone (0.5 pmol/L) and EGF (10 ng/mL). 14-3-3η with dihydrotestosterone and EGF together increased androgen receptor transcriptional activity 6- to 9-fold over 14-3-3η with either dihydrotestosterone or EGF alone. In the same assay, replacing the 14-3-3η sense vector with the control 14-3-3η antisense resulted in ~50% reduction in androgen receptor transactivation.

**Fig. 4.** 
A, 14-3-3η mRNA assayed by quantitative real-time PCR in CWR22 xenograft tumors: intact androgen-stimulated, 6 d postcastration (6 dcx); 6 d postcastration and 48 h after injection of the host mouse with testosterone propionate 0.1 mg (6 dcx + T); 12 d postcastration untreated (12 dcx) and castration-recurrent (CR). B1-4, CWR22 tumors immunostained with anti 14-3-3 antibody sc-731. C1-3, CWR22 tumors stained with 14-3-3η antibody sc-17287 and (C4-6) anti-androgen receptor antibody. B1, C1, C4, CWR22 tumor from intact mouse. B2, C2, C5, CWR22 tumor 6 d postcastration and 48 h after injection of mouse with testosterone propionate 0.1 mg. B3, C3, C6, castration-recurrent CWR22 tumor from mouse 150 d postcastration. Images in B were reduced from ×100 and images in C were obtained with a ×40 lens using the SPOT-4 camera system and reduced from ×400.
Endogenous 14-3-3 and androgen receptor communoprecipitated in CWR-R1 cell lysates using the anti–14-3-3η antibody sc-731, and androgen receptor–14-3-3 complex formation was increased in the presence of dihydrotestosterone (Fig. 3C, middle). However, in the presence of dihydrotestosterone, EGF caused no further increase in communoprecipitation of 14-3-3 with androgen receptor (Fig. 3C, bottom). Immunoprecipitates with anti-actin antibody instead of anti–14-3-3η antibody did not contain androgen receptor. Androgen receptor and 14-3-3 protein levels in CWR-R1 cell lysates are shown by immunoblot analysis (Fig. 3C, top). The results suggest that endogenous 14-3-3η and androgen receptor form a complex in CWR-R1 cells.

Because the native endogenous 14-3-3η seemed to interact with androgen receptor in CWR-R1 cells, we used a dominant negative 14-3-3η to determine the effect of inhibiting endogenous 14-3-3η on androgen receptor transcriptional activation. In transient transfection assays, dominant negative 14-3-3η (DN) inhibited dihydrotestosterone and dihydrotestosterone plus EGF stimulation of MMTV-luciferase transcription (Fig. 3D, left). Moreover, dominant negative 14-3-3η inhibition of the dihydrotestosterone plus EGF stimulated transcriptional activity was prevented by overexpression of 14-3-3η (Fig. 3D, right).

14-3-3η expression is androgen receptor regulated in the CWR22 human prostate cancer xenograft specimens. 14-3-3η mRNA levels measured by quantitative PCR paralleled the changes in 14-3-3η protein detected by immunostaining. Real-time PCR showed a decrease in 14-3-3η mRNA in CWR22 xenografts within 6 days after castration (P < 0.001; Fig. 4A) and an increase within 48 hours following testosterone treatment of the castrated mice (P < 0.01). In CWR22 xenograft tumors that recurred after castration (CR), 14-3-3η mRNA was higher than in tumors from 6-day or 12-day castrate mice and similar to the levels in androgen-stimulated tumors from mice prior to castration (intact).

CWR22 prostate cancer xenograft tumor sections were immunostained with antibody sc-731, which recognizes 14-3-3η and γ isoforms (Fig. 4B), and with sc-17287, specific for 14-3-3η (Fig. 4C). Tumors from androgen-stimulated intact mice exhibited strong 14-3-3 immunostaining with sc-731 (Fig. 4B1). Within 6 days after castration of the host, 14-3-3 levels decreased (Fig. 4B2), and 14-3-3 reappeared within 48 hours after treatment of 6-day castrate mice with androgen (Fig. 4B3), consistent with the increase in RNA and indicating that the expression of 14-3-3η is androgen regulated. In the castration-recurrent CWR22 xenograft tumor, the intensity of 14-3-3η immunostaining increased despite the absence of testicular androgen (Fig. 4B4) and was similar to the levels in CWR22 xenograft tumors from androgen-stimulated mice. Higher magnification images of the CWR22 xenograft tumors stained with 14-3-3η-specific antibody sc-17287 (Fig. 4C1-3) are shown together with androgen receptor (Fig. 4C4-6) in sections from the same tissue blocks. There was strong nuclear localization of 14-3-3η and androgen receptor in the androgen-stimulated (Fig. 4C1 and 4) and castration-recurrent tumors (Fig. 4C3 and 6). In contrast, the 6-day castrate showed substantial loss of 14-3-3η staining in nuclei whereas androgen receptor staining was detected but also weaker than in the intact or castration-recurrent tumors. Earlier studies on androgen receptor expression in the CWR22 xenograft showed a major loss of androgen receptor in nuclei 2 days postcastration with restoration to about 60% of the intact by 6 days postcastration (5).

Thus, during the first several days of androgen deprivation, loss of androgen receptor activity with decreased nuclear localization of androgen receptor was associated with decreased expression and nuclear localization of 14-3-3η. In striking contrast, the castration-recurrent tumor exhibited increased expression of 14-3-3η and nuclear localization with androgen receptor similar to that in androgen-stimulated tumors from intact mice.

Immunostaining of a CWR22 xenograft tumor tissue microarray with the 14-3-3η specific antibody indicated nuclear 14-3-3η levels were decreased by about 50% after 6 and 12 days of androgen deprivation (P < 0.001) and increased within 48 hours of testosterone treatment (P < 0.001; Supplementary Table S1). In castration-recurrent tumors, nuclear levels of 14-3-3η were similar to the levels in tumors from intact mice. 14-3-3η immunostaining in cytoplasm was somewhat weaker and showed little change under the different conditions.

14-3-3η is expressed in clinical specimens of BPH, androgen-dependent and castration-recurrent prostate cancer. 14-3-3η protein was analyzed in clinical specimens of human prostate by immunostaining using the 14-3-3η specific antibody (Santa Cruz, sc-17287, lot #K1803). In Fig. 5 are shown representative sections of androgen-stimulated benign prostate (Fig. 5A1), androgen-stimulated prostate cancer (Fig. 5A2), and castration-recurrent prostate cancer (Fig. 5A3). Immunostaining of tissue microarray sections included groups of 20 specimens each of benign prostatic hyperplasia, androgen-stimulated prostate cancer, and castration-recurrent prostate cancer. Visual scoring of 14-3-3η staining showed no differences (P > 0.05) among the three groups and similar localization in nuclei and cytoplasm. Levels of androgen receptor were also similar in the three groups, but androgen receptor was localized entirely in nuclei (Table 1).

Discussion

Our findings indicate that 14-3-3η promotes androgen receptor transcriptional activation in association with its effects on mitogen signaling. 14-3-3 is known to have a critical role in the Ras-Raf signaling pathway through which growth factors such as EGF activate extracellular signal-regulated kinase 1/2 (ERK1/2) by a phosphorelay from Ras-GTP and Raf1 (18). 14-3-3 maintains Raf-1 in an inactive state in the absence of activation signals but promotes Raf-1 activation and stabilizes its active conformation when signals are received, for example from EGF/hergulin receptors (18). Raf-1 may be activated directly by a protein kinase C-14-3-3η complex supporting another activation mechanism for the mitogen-activated protein (MAP) system in which 14-3-3 isoforms are also phosphorylated (32). Xing et al. (33) showed that dominant negative 14-3-3η inhibited serum-stimulated ERK/MAP kinase (MAPK) activation.

The EGF/hergulin receptors HER1, 2, 3, and 4 are expressed in CWR-R1 cells, and EGF and hergulin activate signaling from these receptors through MAPK, phosphatidylinositol-3-kinase, and AKT (17, 34–36). In CWR-R1 cells EGF was shown to increase phosphorylation of the p160 coactivator TIF2 as well as the interaction between phosphorylated forms of TIF2 and androgen receptor (27). Inhibition of HER-2/new decreased androgen receptor recruitment to androgen response elements (36). LNCap cell growth is increased by the stable transfection of HER2/Neu, and HER2/Neu induces expression of prostate-specific antigen...
through the MAPK pathway (17, 34). 14-3-3η amplification of EGF/heredulin signaling may promote androgen receptor hypersensitivity in prostate cancer (25, 37), and our result showing 14-3-3η enhancement of androgen receptor transcriptional activity at a low level of dihydrotestosterone is consistent with this concept. 14-3-3η increased the transcriptional activity of androgen receptor in LNCaP cells in the absence of androgen and without a detectable interaction with androgen receptor (38).

14-3-3 interactions with androgen receptor may provide a link with mitogen signaling not only through direct effects on androgen receptor but also by modifying androgen receptor coregulators. Zilliacus et al. (31) reported that 14-3-3η binds androgen receptor, glucocorticoid receptor, estrogen receptor α, and estrogen receptor β in cell free pulldown assays. In addition, 14-3-3η bound the nuclear receptor corepressor RIP140 and the coactivator ACTR, a member of the SRC3 family (31) that includes TRAM-1, AIB1, and RAC3 (39). A somewhat weaker interaction was seen with SRC-1 and TIF2 (31). These p160 co-regulators contain LXXLL motifs (40, 41) that mediate binding to activation function 2 (AF2) in the androgen receptor ligand binding domain (42). 14-3-3η was shown to bind a GR fragment containing only the ligand binding domain (31). 14-3-3η contains the motif LXXFL with predicted α-helical secondary structure in the center of its conserved amphipathic groove (18), suggesting it might bind AF2.

Coexpression of 14-3-3η with RIP140 in COS-7 cells promoted the cytoplasmic localization of RIP140, and this effect of keeping RIP140 out of the nucleus was suggested as a mechanism whereby 14-3-3η increased GR transcriptional activity (31). Because RIP140 interacts with androgen receptor and can function as an androgen receptor corepressor (43), the action of 14-3-3η to sequester RIP140 in the cytoplasm might also be a mechanism whereby 14-3-3η enhances the transcriptional activity of androgen receptor. In contrast to its effect on RIP140, we observed under similar experimental conditions in COS-7 cells that 14-3-3η promoted the nuclear localization of androgen receptor in the absence of androgen. Furthermore, a major portion of 14-3-3η was in nuclei together with androgen receptor.

Phosphoserine motifs are established recognition sites for 14-3-3 binding. Numerous proteins bind 14-3-3 through a phosphoserine recognition motif resembling the Raf-1 consensus motif RSxpSxP (18–21, 44, 45). However, variations of the motif have been found where 14-3-3 binding partners have two imperfect sites or sometimes nonphosphorylated acidic motifs. Androgen receptor is a phosphoprotein with several known sites of serine phosphorylation in the NH2-terminal domain and hinge regions (46–49). S650 is in the androgen receptor hinge region close to the nuclear transport signal, and phosphorylation of S650 promotes nuclear export of androgen receptor (47). Some phosphoserines in androgen receptor might be sites for 14-3-3η binding and regulation of androgen receptor function although they are not in perfect consensus motifs. For example, 14-3-3η binding to S650 might possibly promote nuclear retention of androgen receptor by interfering with nuclear export.

In CWR-R1 cells, expression of 14-3-3η enhanced dihydrotestosterone- and EGF-induced androgen receptor transactivation of both the PSA-luciferase and MMTV-luciferase reporter genes. The potentiating effect of 14-3-3η on androgen receptor

| 14-3-3η | Nuclear | 255 ± 11 | Androgen-stimulated | Castration-recurrent prostate cancer |
| 14-3-3η | Cytoplasmic | 246 ± 12 | prostate cancer | prostate cancer |

| Androgen receptor | Nuclear | 212 ± 5 | 225 ± 6 | 220 ± 6 |
| Androgen receptor | Cytoplasmic | 0 | 0 | 0 |
transactivation of MMTV promoter was observed at a low concentration of dihydrotestosterone and to a lesser extent with EGF alone in the absence of dihydrotestosterone, suggesting that 14-3-3ζ may be involved in mitogen activation of androgen receptor by a ligand-independent mechanism. 14-3-3ζ potentiation of androgen receptor transcriptional activity was additive when dihydrotestosterone was combined with EGF. In addition to the 14-3-3ζ regulation of the MMTV and PSA reporter genes reported herein, 14-3-3ζ was reported to enhance androgen-dependent androgen receptor transactivation of the CRISP1 enhancer/promoter in transient transfection assays using the PC3 human prostate cancer cell line (50).

Taken together with the androgen-induced increase in 14-3-3ζ gene expression, 14-3-3ζ enhancement of androgen receptor transcriptional activity suggests there are mutual positive effects of 14-3-3ζ and androgen receptor in prostate cancer cells. Androgen dependence of 14-3-3ζ expression in the CWR22 xenograft was shown by a decline in both protein and mRNA within 6 days following castration of tumor-bearing mice and an increase in 14-3-3ζ expression in response to testosterone treatment of 6-day castrate mice. 14-3-3ζ mRNA was also higher in castration-recurrent CWR22 xenograft tumors than in tumors from the castrate mice. This increase in expression of 14-3-3ζ in the castration-recurrent CWR22 tumor is characteristic of androgen-regulated genes in this model system (7), including the EGF receptor (51), and is consistent with the presence of a functionally active androgen receptor in the recurrent CWR22 xenograft despite the absence of circulating testicular androgens. The relationship between 14-3-3ζ levels in androgen-stimulated and castration-recurrent CWR22 xenografts is similar to that in clinical specimens of androgen-stimulated and castration-recurrence cancers as noted by immunohistochemical analysis of 14-3-3ζ in tissue microarray sections. Local tissue production of androgens that activate androgen receptor may promote the expression of 14-3-3ζ in clinical castration-recurrence cancer (12) and possibly in the CWR22 xenograft model as well. Androgen upregulation of another 14-3-3 isoform, similar to 14-3-3ζ, was found in a proteomic analysis of LNCaP prostate cancer cell protein lysates (52). Similarly, dihydrotestosterone stimulated an increase in 14-3-3ζ protein in M12 cells, an androgen receptor–positive cell line derived from SV40 T-antigen immortalized nonneoplastic human prostate epithelial cells (53).

14-3-3ζ is a heat shock protein regulated by heat stress in Drosophila S2 cells (54) consistent with the heat shock protein–like functions of 14-3-3 in cell survival (55, 56). The stress-induced heat shock factor (HSF1), a potent transcription factor for Hsp70 and Hsp90, was shown to interact with its response element in the promoter region of the 14-3-3ζ gene, suggesting that HSF1 mediated the stress-induced transcription of 14-3-3ζ (54). HSF1 protein was shown by Western blot to be present in several prostate cancer cell lines. In addition, immunostaining of HSF1 protein was higher in prostate carcinoma cells than in normal prostate epithelial cells (57), suggesting that HSF1 is a positive selection factor in prostate cancer as a consequence of its role in promoting cell survival. These findings, together with observations that 14-3-3 levels are increased by androgen, raise the possibility that HSF1 expression or activation might be increased by androgen and mediate the androgen-induced increase in 14-3-3.

A potential link between androgen activation of androgen receptor and HSF1 was suggested with the discovery that a highly conserved sequence in the androgen receptor NH2-terminal region is a binding site for CHIP (COOH-terminus of Hsp70-interacting protein; refs. 58, 59). Hsp70 forms a stable complex with the transactivation domain of HSF1 and thereby inhibits HSF1 transcriptional activity. CHIP activates HSF1 by releasing it from Hsp70 inhibition. Androgen receptor activation by androgen binding drives androgen receptor into the nucleus and could dissociate CHIP from androgen receptor. CHIP would then be free to activate HSF1 and increase activated HSF1 within the nucleus where it could stimulate 14-3-3 gene transcription.

The multifunctional nature of 14-3-3ζ suggests it may influence numerous signaling mechanisms in prostate cancer cells. Our studies indicate that 14-3-3ζ has a role in promoting the concerted actions of androgen receptor and EGF in castration-recurrent prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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