Published in final edited form as:
Clin Cancer Res. 2009 December 15; 15(24): 7571–7581. doi:10.1158/1078-0432.CCR-08-1976.

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

Mark A. Titus[2,6,7,*], **Jiann-an Tan**[2], **Christopher W. Gregory**[2,4,6,§], **O. Harris Ford**[6], **Romesh R. Subramanian**[9,#], **Haian Fu**[9], **Elizabeth M. Wilson**[2,5,6], **James L. Mohler**[3,4,6,7,8], and **Frank S. French**[2,6,*]

[2]Department of Pediatrics (Laboratories for Reproductive Biology), University of North Carolina School of Medicine, Chapel Hill, NC 27599-7500

[3]Department of Surgery (Division of Urology) University of North Carolina School of Medicine, Chapel Hill, NC 27599-7500

[4]Department of Pathology and Laboratory Medicine, University of North Carolina School of Medicine, Chapel Hill, NC 27599-7500

[5]Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, NC 27599-7500

[6]Lineberger Comprehensive Cancer Center, University of North Carolina School of Medicine, Chapel Hill, NC 27599-7500

[7]Department of Urologic Oncology, Roswell Park Cancer Institute, Buffalo, NY 14263

[8]Department of Urology, University at Buffalo School of Medicine and Biotechnology, Buffalo, NY 14263

[9]Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

Abstract

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

Experimental Design and Results—AR and 14-3-3η co-localized in COS cell nuclei with and without androgen and 14-3-3η promoted AR nuclear localization in the absence of androgen. 14-3-3η interacted with AR in cell-free binding and coimmunoprecipitation assays. In the recurrent human CaP cell line, CWR-R1, native endogenous AR transcriptional activation was stimulated by

*To whom correspondence should be addressed: Frank S. French, M.D., Department of Pediatrics (Laboratories for Reproductive Biology), CB#7500, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599-7500, phone 919-966-0930; Fax 919-966-2203; fsfrench@med.unc.edu and Mark A. Titus, Ph.D., Department of Urologic Oncology, Roswell Park Cancer Institute; Buffalo, NY 14263: phone 716-845-4876; Mark.Titus@roswellpark.org.
§Present Address: Clinsys Clinical Research, 5840 Colonnade Center Drive, Suite 501, Raleigh, NC 27615
#Pfizer Target Biology, Pfizer Research Technology Center, Cambridge MA 021390

Statement of Translational Relevance We demonstrate that 14-3-3η is an androgen receptor (AR) coactivator in post-castration recurrent prostate cancer (CR-CaP) through its direct interaction with AR and by augmenting mitogen effects on AR transcriptional activity. The use of AR siRNA technology in the experimental setting as well as in recent phase 1 clinical trials with a CYP17 inhibitor of androgen production provide strong evidence that AR action is central to the growth of CR-CaP. Our studies identify a new potential target to inhibit CR-CaP growth. We demonstrate that 14-3-3η causes AR to be nuclear in the absence of androgen, whereas without 14-3-3η, AR is cytoplasmic. We demonstrate also that AR signaling increases the production of 14-3-3η through the synthesis of new mRNA and that 14-3-3η levels remain elevated together with AR in CR-CaP. Studies described in this report provide a potential new target for combination therapy to block the AR driven growth of CR-CaP.

14-3-3η at low DHT concentrations and was increased by EGF. Moreover, the DHT and EGF dependent increase in AR transactivation was inhibited by a dominant negative 14-3-3η. In the CWR22 CaP xenograft model, 14-3-3η expression was increased by androgen, suggesting a feed-forward mechanism that potentiates both 14-3-3η and AR 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 AR in nuclei and the similar amounts expressed in castration-recurrent CaP, androgen-stimulated CaP and benign prostatic hyperplasia were consistent with AR activation in recurrent CaP.

Conclusion—14-3-3η enhances androgen and mitogen induced AR transcriptional activity in castration-recurrent CaP.

Keywords

androgen receptor (AR); androgen; dihydrotestosterone; 14-3-3η; EGF; gene transcription; prostate cancer

Introduction

The androgenic steroid hormones, testosterone and its more active metabolite dihydrotestosterone (DHT), maintain and stimulate 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 since prostate cancer recurs (referred to as castration-recurrent prostate cancer) and almost always causes death. The expression of AR (2–6) and androgen-regulated genes (7,8) in castration-recurrent cancer implicates AR signaling in recurrent growth despite low levels of circulating androgen. Potential mechanisms include AR gene amplification (9), gain of function mutations in the AR gene resulting in promiscuous ligand binding (6,10, see also www.androgendb.mcgill.ca), coactivator overexpression (11), intracrine production of testosterone and DHT (12,13) and enhanced activation of AR through mitogen signaling (14–17).

The 14-3-3 family of homo or heterodimeric α-helical proteins interact with a variety of signaling proteins including kinases, phosphatases, transcription factors and non-kinase 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, however 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 (24,7) 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 demonstrate that 14-3-3η (eta) is a human AR binding protein in CWR-R1 cells (25) and that 14-3-3η binding to AR is associated with increased AR transcriptional activity that is enhanced by epidermal growth factor (EGF) and DHT. 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 14-3-3η similar to the amounts expressed in androgen-stimulated benign prostate and androgen-stimulated prostate cancer. The results indicate that AR regulates the expression of 14-3-3η and that AR regulation is active in castration-recurrent prostate cancer. 14-3-3η localizes with AR in nuclei and provides a link between EGF signaling and AR transcriptional activation in prostate cancer.

Material and Methods

Plasmid construction

Full-length 14-3-3η (18) was excised from the vector pPCR-Script Amp 14-3-3η using EcoR1 and Sac1. The excised DNA was blunt ended with Klenow enzyme (Life Technologies, Inc. Rockville, MD), and ligated into the blunt ended BamH1 site of pSG5 (Stratagene, La Jolla, CA). 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 14-3-3η cDNA (pcDNA3.1-myc-14-3-3η-R56, 60A) was obtained from Andrey S. Shaw, Washington University, St. Louis, MO.

Solid-phase binding assays

Purified full-length human hexahistidine-tagged 14-3-3η or 14-3-3ζ (zeta) was incubated with Ni+2-charged Sepharose 6B beads (Novagen, San Diego, CA) for 1 hour at 4°C. Radiolabeled AR was generated using the TNT in vitro transcription-translation system (Promega, Madison, WI). The full-length DNA template pSG5-AR was incubated with TNT rabbit reticulocyte lysates in the presence of ^{35}S-methionine. For the binding assays (26), the immobilized 14-3-3η or 14-3-3ζ protein (1 μg each) was mixed with ^{35}S-labeled AR in Nonidet P-40 buffer (1% Nonidet P-40, 137 mM NaCl, 1 mM $MgCl_2$, and 40 mM Tris-HCl (pH 8.0) for 1 hour at 4°c with rotation. 14-3-3η or 14-3-3ζ complexes were washed 3 times with Nonidet P-40 buffer and 3 times with radioimmune precipitation assay wash (1% Nonidet P-40, 137 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 20 mM Tris-HCL (pH 8.0)), and boiled 5 min in 2x SDS sample buffer before resolution by SDS-PAGE (12%). Gels were dried and autoradiography performed with BioMax film (Eastman Kodak Co. Rochester, NY) at −80° C. Image analysis was performed using the Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation and immunoblot analysis

Protein lysates were prepared from COS cells grown on 10 cm culture dishes and cotransfected with sense or antisense 14-3-3η and human AR 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 precleared with mouse IgG and protein A-agarose. To examine AR/14-3-3η complex formation in COS cell and CWRR1 cell lysates, anti-14-3-3 antibody that recognizes η and γ isoforms (Santa Cruz Biotechnology Inc., Santa Cruz, CA, sc-731) or anti-actin antibody (Research Diagnostic Inc., Flanders, NJ) were incubated with rotation for 1 hour followed by the addition of protein A-agarose with constant rotation at 4°C overnight. Immunoblot analysis was performed using AR monoclonal antibody (Biogenex, San Ramon, CA) at 1:5000 dilution and secondary antibody (horseradish-peroxidase conjugated anti-

mouse, Promega Corporation, Madison, Wis.) at 1:10000 dilution. Antigen-antibody complexes were detected using enhanced chemiluminescence (DuPont, NEN Research Products, Boston, MA).

Prostate cancer specimens were pulverized in liquid nitrogen and mixed with 0.5 mL of radioimmunoprecipitation assay buffer containing Complete™ protease inhibitors (Roche, Indianapolis, IN) (11). Tissue was homogenized for 30 sec on ice, incubated for 45 min on ice and centrifuged at 10000 × 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 performed as described above using 14-3-3η antibody specific for the η isoform (Santa Cruz sc-17287).

Transient cotransfection assays

Cotransfection assays using monkey kidney epithelial CV1 cells were performed 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% CO_2 in DMEM-H medium containing 2 mM L-glutamine, 10% bovine calf serum, 20 mM HEPES, 100 units/mL of penicillin and 100 μg/mL streptomycin. On the day before transfection, 4.5×10^6 cells per 6 cm culture dish were grown in the same medium for about 20 hours until 70% confluent. 0.25 μg probasin-luciferase vector, 0.25 μg human AR expression vector (pSG5-hAR), and 0.5 μg of pSG5-14-3-3η sense or pSG5-14-3-3η antisense vector per 6 cm culture dish were cotransfected using the Effectene (Qiagen, Valencia, CA) method. After incubation for 24 hours the medium was changed to DMEM-H without phenol red plus 5% charcoal stripped serum in the presence or absence of steroid and the cells were incubated for 40 hours.

CWR-R1 cells were maintained in prostate growth medium [DMEM-H (Gibco/Invitrogen) containing linoleic acid 0.1 μg/mL, L-glutamine 2 mM, nicotinamide 1.2 μg/mL, insulin 5 μg/mL, transferrin 5 μg/mL, selenium 5 ng/mL, 100 units/mL penicillin, 100 μg/mL streptomycin, 20 mM HEPES, pH 7.2, FBS 2% and EGF (mouse, BD Biosciences) 10 ng/mL]. Transient transfections were performed as described (27) with minor modifications. CWR-R1 cells were plated the day before at 10^6 cells per 6 cm culture dish in prostate growth medium without EGF and grown for about 20 hours 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 pSG5-14-3-3η sense or 14-3-3η antisense using the Effectene method. CWR-R1 cells were cotransfected similarly with MMTV-luciferase reporter (0.5 μg) and 250 ng pSG5-14-3-3η sense or antisense. CWR-R1 transfection medium was replaced with phenol red-free, serum-free medium (27, Improved MEM Zinc Option; Invitrogen, Carlsbad, CA) with or without addition of DHT or EGF as indicated and incubated 24 hours.

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 standard deviation of optical units from 3 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 Protocol Review Committee, Institutional Review Board, Federal Health Insurance Portability Accountability Act (HIPAA) and Protected Health Information (PHI) Regulations. CWR22 human prostate cancer xenografts were propagated and procured as described (7,23,27) in

compliance with approved protocols and regulations of the University of North Carolina School of Medicine at Chapel Hill IACUC committee.

Immunostaining of tissue sections

Formalin-fixed, paraffin-embedded xenograft tumors were sectioned and stained using standard immunohistochemical techniques. *Method for Fig. 4 panel B, 1–4*: After being deparaffinized and rehydrated, tissue sections (8 μm) were incubated in epitope-retrieval buffer (AntigenDecloaker, pH 6.0; Biocare Medical, Walnut Creek, CA) 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, Burlingame, CA). Rabbit polyclonal anti-14-3-3η antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 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 performed with hematoxylin. *Method for Fig. 4 panel C, 1–6*: tissue sections (8 μm) were incubated in 83% methanol and 5% H_2O_2 at room temperature for 30 min to reduce endogenous peroxidase activity, followed by exposure to 0.01 M sodium citrate, pH 6.0 for 15 min in a microwave oven at high setting. For 14-3-3η detection using goat polyclonal anti-14-3-3η antibody (Santa Cruz Biotechnology, Santa Cruz, CA sc-17287, lot #K1803), sections were blocked with 1.5% normal rabbit serum and incubated overnight at 4°C in a humidified chamber with 14-3-3η 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 antigoat IgG, immunostaining was amplified using the Vector Laboratories Elite ABC Kit and the immunoperoxidase complex was visualized using diaminobenzidine (DAB). Sections were exposed to osmium vapors and counterstained with 0.05% toluidine blue in 30% ethanol, dehydrated, cleared in xylene and mounted with Permount (Fisher). For AR immunostaining using rabbit polyclonal anti-AR-PG21 (Upstate USA, Inc., Lake Placid, NY cat. #06-680), 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 DAB step to the end of the staining protocol. Photographs (40X) were taken using a SPOT-4 Megapixel Digital Color Camera System (Diagnostic Instruments, Inc., Sterling Heights, MI) 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 14-3-3η specific antibody sc-17287, lot #K1803. Tissue microarray sections were visually scored by three individuals without knowledge of tumor or patient status. Epithelial cell 14-3-3η 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.

Immunofluoresence microscopy

Assays were performed using COS cells as described (30). Cells were grown in DMEM-H (Gibco/Invitrogen) containing 2 mM L-glutamine, 20 mM HEPES buffer pH 7.2, 100 units/mL penicillin, 100 μg/mL streptomycin and 5% bovine calf serum and 10,000 cells/well were seeded on coverslips (Microscope coverglass, Fischer Scientific, Pittsburg, PA) in 12-well plates and cultured in the same medium. The following day, DEAE-dextran followed by glycerol shock was used to transfect 1 μg each of pCMV-AR, pSG FLAG-14-3-3η, 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 nM DHT and in the absence

of serum or phenol red. Media were changed the following day and incubations continued for 24 hours. Incubations with primary antibodies (anti-AR 1:250, and/or anti-FLAG 1:1000) were for 1 hour at room temperature. After washing 3 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-AR ab3510 was obtained from Abcam, Cambridge, MA; mouse anti-FLAG antibodies from Sigma. Fluorescein (FITC)-conjugated, AffiniPure Donkey Anti-rabbit IgG and Rhodamine (TRITC)-conjugated AffiniPure Donkey Anti-mouse IgG were from Jackson Immuno Research, West Grove, PA.

Comparative (C_t method) quantitative real-time PCR analysis

Genomic DNA-free total RNA was isolated from archived CWR22 tumors (n=2) from mice that were intact, 6-days and 12-days after castration, and recurrent tumors from mice 150-days after castration. TRIzol reagent (Gibco, Rockville, MD) was used according to the manufacturer's protocol. Total RNA integrity was determined using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). 14-3-3η mRNA levels were assayed using beta-glucuronidase (GUS) as calibrator in comparative real-time PCR. PCR was performed in a one step reaction using 50 ng total RNA and 1 unit of reverse transcriptase (Roche, Indianapolis, IN) in a total volume of 30 μL [48°C, 30 min; 95°C, 10 min; 40 cycles at 95°C, 15 sec; 63°C, 1 min] using the Applied Biosystems RT-PCR 7500 System (Foster City, CA)]. Specific primers for amplifying 14-3-3η and GUS were based on human GenBank sequences. Forward and reverse primer sequences were 14-3-3η, 5'-ttggtgccaggcgatctt and 5'-ccaatttcttttcgtttccatca; GUS, 5'-tggtgctgaggattggca and 5'-tagcgtgtcgaccccattc.

Statistical analyses

Mean nuclear and cytoplasmic 14-3-3η immunostaining visual scores from human and CWR22 tissue microarrays, mean 14-3-3η mRNA levels in CWR22 tumors and mean densitometry values for solid phase binding assays were calculated and statistical analysis performed using Statgraphics Plus 4.1 (Manugistics, Inc., Rockville, MD). The Mann-Whitney comparison test and students t-test were used and differences were considered significant at $P < 0.05$.

Results

14-3-3η interacts with AR

His-tag pull down experiments were performed to test for a direct interaction between AR and 14-3-3η protein. Binding of *in vitro* translated ^{35}S-labeled AR to His-tag 14-3-3η was positive in the presence of 1 μM DHT compared to the Ni-Sepharose control (P value = 0.013). There was no difference between control and the minus DHT (Fig. 1A). These results are in agreement with an earlier report, Zilliacus et al. (31) who showed that AR bound GST-14-3-3η in the presence of 1 μM testosterone. In addition, we found that under these conditions AR did not bind the full-length 14-3-3ζ isoform, either in the presence or absence of DHT suggesting a certain isoform selectivity for AR regulation. Whether AR favors the 14-3-3η isoform within the 14-3-3 family of proteins remains to be established.

Coimmunoprecipitation studies were performed to test the interaction between AR and 14-3-3η. COS cells were cotransfected with vectors that express recombinant human full-length wild type AR and either sense or antisense 14-3-3η expression vector DNA. Lysate proteins were immunoprecipitated with anti-14-3-3 antibody (Santa Cruz sc-731 that recognizes η and γ isoforms) and immunoblotted with AR antibody. AR co-immunoprecipitated with 14-3-3η in lysates from COS cells cotransfected with sense 14-3-3η but not with antisense 14-3-3η (Fig. 1B). The amount of AR complexed with 14-3-3η was greater in the presence of DHT while the expression levels (input) of AR and 14-3-3η were similar (Fig. 1C). The AR/14-3-3η association appeared specific in that

immunoprecipitates with anti-actin instead of anti-14-3-3η antibody did not contain AR. While the interaction between recombinant wild-type AR and 14-3-3η was stronger in the presence of DHT (Fig. 1B), complex formation in the absence of DHT suggested the interaction was not completely androgen dependent.

When recombinant 14-3-3η and AR were expressed separately in COS cells, AR was mainly cytoplasmic without DHT and nuclear with DHT while 14-3-3η was both nuclear and cytoplasmic without or with DHT (Fig.1C, *upper panel*). Co-expression of 14-3-3η and AR in the absence of DHT resulted in major nuclear localization of AR and co-localization with 14-3-3η in nuclei (Fig. 1C, *lower panel*). In contrast, an AR corepressor, CR6 interacting factor-1 (CRIF) localized in nuclei both in the presence and absence of DHT (Fig. 1C, upper panel) and when co-expressed with AR in the absence of DHT (Fig. 1C, *lower panel*) CRIF did not alter the subcellular partitioning of AR. In the presence of DHT, the strong nuclear localization of AR was not altered by co-expression of 14-3-3η or CRIF.

14-3-3η enhances androgen dependent AR transactivation

To examine the effect of 14-3-3η on the transcriptional function of AR, transient cotransfection assays were performed in CV-1 cells. In cells transfected with AR together with a probasin-luciferase reporter gene and the sense 14-3-3η vector, 1 nM DHT increased luciferase activity 3–4 fold greater than in control cells transfected with the antisense 14-3-3η (Fig. 2). In the absence of DHT, basal luciferase activity in cells transfected with sense 14-3-3η was only slightly higher than in cells transfected with antisense 14-3-3η. In these experiments, the antisense 14-3-3η vector DNA was used to balance the sense 14-3-3η vector DNA. The results indicate that 14-3-3η amplifies DHT induced AR transactivation in CV1 cells.

To investigate the ligand specificity of 14-3-3η stimulation of AR transcriptional activation, DHT was compared with progesterone, estradiol and the antiandrogen hydroxyflutamide. In CV1 cell cotransfection assays using the probasin-luciferase reporter gene with DHT, luciferase activity with sense 14-3-3η was greater than with antisense 14-3-3η. 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 nM (not shown) suggesting that at the tissue steroid levels found in BPH and prostate cancer (12) it is androgen induced AR transactivation that is amplified by 14-3-3η.

14-3-3η enhances EGF and DHT induced AR transactivation in a castration-recurrent prostate cancer cell line

We found in earlier studies that EGF increases DHT dependent AR transcriptional activation in CWR-R1 cells (27). The EGF effect on AR action involved phosphorylation and increased coactivation by TIF2/GRIP1. Since 14-3-3 has a propensity for binding to phosphorylated proteins, we reasoned it might have a role in EGF amplification of AR transactivation. With this in mind, the effect of 14-3-3η on EGF and DHT induced AR transactivation was determined in the CWR-R1 cell line. In the presence of the 14-3-3η sense vector, 0.1 nM DHT increased androgen dependent transcriptional activation of the PSA-luciferase reporter gene about 5-fold above the no DHT control (Fig. 3A). Concurrent addition of 100 ng/mL EGF and 0.1 nM DHT further increased AR transcriptional activity 2-fold over that of DHT alone. AR transactivation in the presence of DHT 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 AR 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 AR transactivation in the presence of 14-3-3η was observed at lower concentrations of DHT (0.5 pM) and EGF (10 ng/mL).

14-3-3η with DHT and EGF together increased AR transcriptional activity 6 to 9 fold over 14-3-3η with either DHT 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 AR transactivation.

Endogenous 14-3-3 and AR coimmunoprecipitated in CWR-R1 cell lysates using the anti-14-3-3η antibody sc-731 and AR-14-3-3 complex formation was increased in the presence of DHT (Fig. 3C *middle panel*). However in the presence of DHT, EGF caused no further increase in coimmunoprecipitation of 14-3-3 with AR (Fig. 3C, *lower panel*). Immunoprecipitates with anti-actin antibody instead of anti-14-3-3η antibody did not contain AR. AR and 14-3-3 protein levels in CWR-R1 cell lysates are shown by immunoblot analysis (Fig. 3C, *upper panel*). The results suggest that endogenous 14-3-3η and AR form a complex in CWR-R1 cells.

Since the native endogenous 14-3-3η appeared to interact with AR in CWR-R1 cells, we used a dominant negative 14-3-3η to determine the effect of inhibiting endogenous 14-3-3η on AR transcriptional activation. In transient transfection assays, dominant negative 14-3-3η (DN) inhibited DHT and DHT+EGF stimulation of MMTV-luciferase transcription (Fig. 3D, left panel). Moreover, dominant negative 14-3-3η inhibition of the DHT+EGF stimulated transcriptional activity was prevented by over expression of 14-3-3η (Fig. 3D, right panel).

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. RT-PCR demonstrated a decrease in 14-3-3η mRNA in CWR22 xenografts within 6 days after castration, P value < 0.001 (Fig. 4 *panel A*) and an increase within 48 hours following testosterone treatment of the castrated mice, P value < 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, that recognizes 14-3-3η and γ isoforms (Fig. 4 *panel B*), and with sc-17287, specific for 14-3-3η (Fig. 4 *panel C*). Tumors from androgen-stimulated intact mice exhibited strong 14-3-3 immunostaining with sc-731 (B-1). Within 6 days after castration of the host, 14-3-3 levels decreased (B-2) and 14-3-3 reappeared within 48 hours after treatment of 6-day castrate mice with testosterone (B-3) 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 (B-4) 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, lot #K1803 (Fig. 4 *panel C*, 1–3) are shown together with AR (Fig. 4 *panel C*, 4–6) in sections from the same tissue blocks. There was strong nuclear localization of 14-3-3η and AR in the androgen-stimulated (Fig. 4 *panel C*, 1 and 4) and castration-recurrent tumors (Fig. 4 *panel C*, 3 and 6). In contrast, the 6-day castrate showed substantial loss of 14-3-3η staining in nuclei while AR staining was detected but also weaker than in the intact or castration-recurrent tumors. Earlier studies on AR expression in the CWR22 xenograft demonstrated a major loss of AR in nuclei 2 days post-castration with restoration to about 60% of the intact by 6 days post-castration (5). Thus, during the first several days of androgen deprivation, loss of AR activity with decreased nuclear localization of AR 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 AR 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 values < 0.001) and increased within 48 hours of testosterone treatment, P values < 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.

14-3-3 η 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. 5 A1), androgen-stimulated prostate cancer (Fig. 5 A2) and castration-recurrent prostate cancer (Fig. 5 A3). 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 value > 0.05) among the three groups and similar localization in nuclei and cytoplasm. Levels of AR were also similar in the three groups, however AR was localized entirely in nuclei (Table 1).

Discussion

Our findings indicate that 14-3-3η promotes AR 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 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/heregulin receptors (18). Raf-1 may be activated directly by a PKC-14-3-3η complex supporting another activation mechanism for the MAP system in which 14-3-3 isoforms are also phosphorylated (32). Xing et al. (33) demonstrated that dominant negative 14-3-3η inhibited serum stimulated ERK/MAPK activation.

The EGF/heregulin receptors HER1, 2, 3, and 4 are expressed in CWR-R1 cells and EGF and heregulin 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 co-activator TIF2 as well as the interaction between phosphorylated forms of TIF2 and AR (27). Inhibition of HER-2/neu decreased AR 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 MAP kinase pathway (17,34). 14-3-3η amplification of EGF/heregulin signaling may promote AR hypersensitivity in prostate cancer (25,37) and our result showing 14-3-3η enhancement of AR transcriptional activity at a low level of DHT is consistent with this concept. 14-3-3σ (sigma) increased the transcriptional activity of AR in LNCaP cells in the absence of androgen and without a detectable interaction with AR (38).

14-3-3 interactions with AR may provide a link with mitogen signaling not only through direct effects on AR but also by modifying AR coregulators. Zilliacus et al. (31) reported that 14-3-3η binds AR, GR, ERα and ERβ in cell free pull-down 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 coregulators contain LXXLL motifs (40,41) that mediate binding of the coregulators to activation function 2 (AF2) in the AR 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 activation (31). Since RIP140 interacts with AR and can function as an AR 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 AR. 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 AR in the absence of androgen. Furthermore, a major portion of 14-3-3η was in nuclei together with AR.

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 non-phosphorylated acidic motifs. AR is a phosphoprotein with several known sites of serine phosphorylation in the NH_2-terminal domain and hinge regions (46–49). S650 is in the AR hinge region close to the nuclear transport signal and phosphorylation of S650 promotes nuclear export of AR (47). Some phosphoserines in AR might be sites for 14-3-3η binding and regulation of AR function even though they are not in perfect consensus motifs. For example, 14-3-3η binding to pS650 might possibly promote nuclear retention of AR by interfering with nuclear export.

In CWR-R1 cells, expression of 14-3-3η enhanced DHT and EGF induced AR transactivation of both the PSA-luciferase and MMTV-luciferase reporter genes. The potentiating effect of 14-3-3η on AR transactivation of MMTV promoter was observed at a low concentration of DHT and to a lesser extent with EGF alone in the absence of DHT suggesting that 14-3-3η may be involved in mitogen activation of AR by a ligand independent mechanism. 14-3-3η potentiation of AR transcriptional activity was additive when DHT was combined with EGF. In addition to the 14-3-3η regulation of the MMTV and PSA reporter genes reported herein, 14-3-3η was reported to enhance androgen dependent AR transactivation of the CRISP-1 enhancer/promoter in transient transfection assays using the PC3 human prostate cancer cell line (50).

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

protein in M12 cells, an AR positive cell line derived from SV40 T-antigen immortalized non-neoplastic human prostate epithelial cells (53).

14-3-3 ζ (zeta) 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 AR and HSF1 was suggested with the discovery that a highly conserved sequence in the AR NH_2-terminal region is a binding site for CHIP (C-terminus of Hsp70-interacting protein) (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. AR activation by androgen binding drives AR into the nucleus and could dissociate CHIP from AR. 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 14-3-3 suggests it may influence numerous signaling mechanisms in prostate cancer cells. Our studies indicate that 14-3-3η has a role in promoting the concerted actions of AR and EGF in castration-recurrent prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Sharon Campbell, Department of Biochemistry and Biophysics and Dr. Channing Der, Department of Pharmacology, UNC-Chapel Hill School of Medicine for providing purified his-tagged 14-3-3ζ and vectors, and Dr. Andrey S. Shaw, Washington University and Howard Hughes Medical Institute, St. Louis, Mo. for providing the dominant negative 14-3-3η R56,60A mutant. Dr. Peter Petrusz, Director of the Laboratories for Reproductive Biology, Molecular Histology Core for helpful advice and Mrs. Gail Grossman for excellent technical assistance.

This work was supported by NIH Grants HD-07315 (to MAT), HD-04466 (FSF), HD-16910 (EMW), CA-77739 (FSF, JLM, EMW); United States Army Medical Research and Material Command Grants DAMD17-00-1-009 and DAMD17-02-1-0110 (CWG, EMW) and NICHD/NIH through cooperative agreement U54-HD35041 as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research.

References

1. Denmeade SR, Lin XS, Isaacs JT. Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. Prostate 1996;28:251–65. [PubMed: 8602401]
2. de Vere White R, Meyers F, Chi SG, et al. Human androgen receptor expression in prostate cancer following androgen ablation. Eur Urol 1997;31:1–6. [PubMed: 9032526]
3. Culig Z, Hobisch A, Hittmair A, et al. Expression, structure, and function of androgen receptor in advanced prostatic carcinoma. Prostate 1998;35:63–70. [PubMed: 9537601]
4. Hobisch A, Culig Z, Radmayr C, et al. Distant metastases from prostatic carcinoma express androgen receptor protein. Cancer Res 1995;55:3068–72. [PubMed: 7541709]

5. Kim D, Gregory CW, French FS, Smith GW, Mohler JL. Androgen receptor expression and cellular proliferation during transition from androgen dependent to recurrent growth in the CWR22 Prostate Cancer Xenograft. Am J Pathol 2002;160:219–26. [PubMed: 11786415]
6. Heinlein CA, Chang C. Androgen receptor in prostate cancer. Endocrine Reviews 2004;25:276–308. [PubMed: 15082523]
7. Gregory CW, Hamil KG, Kim D, et al. Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. Cancer Res 1998;58:5718–24. [PubMed: 9865729]
8. Mousses JL, Wagner U, Chen Y, et al. Failure of hormone therapy in prostate cancer involves systemic restoration of androgen responsive genes and activation of rapamycin sensitive signaling. Oncogene 2001;20:6718–23. [PubMed: 11709706]
9. Visakorpi T, Hytinen E, Koivisto P, et al. In vivo amplification of the androgen receptor gene in progression of human prostate cancer. Nat Genet 1995;9:401–6. [PubMed: 7795646]
10. Tan J-A, Sharief Y, Hamil KG, et al. Dehydroepiandrosterone activates mutant androgen receptor expressed in the androgen-dependent human prostate cancer xenograph CWR22 and LNCaP cells. Mol Endocrinol 1997;11:450–9. [PubMed: 9092797]
11. Gregory CW, He B, Johnson RT, et al. A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. Cancer Res 2001;61:4315–9. [PubMed: 11389051]
12. Mohler JL, Gregory CG, Ford OH, et al. The androgen axis in recurrent prostate cancer. Clin Cancer Res 2004;10:440–48. [PubMed: 14760063]
13. Titus MA, Schell MJ, Lih FB, Tomer KB, Mohler JL. Testosterone and dihydrotestosterone tissue levels in recurrent prostate cancer. Clin Cancer Res 2005;11:4653–7. [PubMed: 16000557]
14. Grossman ME, Huang H, Tindall DJ. Androgen receptor signaling in androgen refractory prostate cancer. J. Nat. Cancer Inst 2001;93:1687–97. [PubMed: 11717329]
15. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nat Rev Cancer 2001;1:34–45. [PubMed: 11900250]
16. Arnold JT, Isaacs JT. Mechanisms involved in the progression of androgen-independent prostate cancers: it is not only the cancer cell's fault. Endocrine-Related Cancer 2002;9:61–73. [PubMed: 11914183]
17. Scher HI, Sawyers Cl. Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. J Clin Oncol 2005;23:8253–61. [PubMed: 16278481]
18. Fu H, Subramanian RR, Masters SC. 14-3-3 Proteins: Structure, function, and regulation. Ann Rev Pharmacol Toxicol 2000;40:617–47. [PubMed: 10836149]
19. Yaffe MB, Elia AEH. Phosphoserine/threonine-binding domains. Curr Opin Cell Biol 2001;13:131–38. [PubMed: 11248545]
20. Muslin AJ, Xing H. 14-3-3 proteins: Regulation of subcellular localization by molecular interference. Cell Signal 2000;12:703–9. [PubMed: 11152955]
21. Tzivion G, Shen YH, Zhu J. 14-3-3 proteins; bringing new definitions to scaffolding. Oncogene 2001;20:6331–38. [PubMed: 11607836]
22. Hermeking H. The 14-3-3 cancer connection. Nat Rev Cancer 2003;3:931–43. [PubMed: 14737123]
23. Wainstein MA, He F, Robinson D, et al. CWR22: Androgen-dependent xenograft model derived from a primary human prostatic carcinoma. Cancer Res 1994;54:6049–52. [PubMed: 7525052]
24. Myers RB, Oelschlager DK, Coan PN, et al. Changes in cyclin dependent kinase inhibitors p21 and p27 during the castration induced regression of the CWR22 model of prostatic adenocarcinoma. J Urol 1999;161:945–9. [PubMed: 10022731]
25. Gregory CW, Johnson RT, Mohler JL, French FS, Wilson EM. Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen. Cancer Res 2001;61:2892–8. [PubMed: 11306464]
26. Zhang L, Wang H, Liu D, Liddington R, Fu H. Raf-1 kinase and exoenzyme S interact with 14-3-3 zeta through a common site involving lysine 49. J Biol Chem 1997;272:13717–24. [PubMed: 9153224]
27. Gregory CW, Fei X, Ponguta LA, et al. Epidermal growth factor increases coactivation of the androgen receptor in recurrent prostate cancer. J Biol Chem 2004;279:7119–30. [PubMed: 14662770]

28. Titus MA, Gregory CW, Ford OH 3rd, et al. Steroid 5alpha-reductase isozymes I and II in recurrent prostate cancer. Clin Cancer Res 2005;11:4365–71. [PubMed: 15958619]
29. Miyamoto KK, McSherry SA, Dent GA, et al. Immunohistochemistry of the androgen receptor in human benign and malignant prostate tissue. J Urol 1993;149:1015–9. [PubMed: 7683339]
30. Bai S, He B, Wilson EM. Melanoma antigen gene protein MAGE-11 regulates androgen receptor function by modulating the interdomain interaction. Mol Cell Biol 2005;25:1238–57. [PubMed: 15684378]
31. Zilliacus J, Holter E, Wakui H, Tazawa H, Treuter E, Gustafsson JA. Regulation of glucocorticoid receptor activity by 14-3-3 dependent intracellular relocalization of the corepressor RIP140. Mol Endocrinol 2001;15:501–11. [PubMed: 11266503]
32. Aitken A. 14-3-3 proteins: A historic overview. Seminars in Cancer Biology 2006;16:162–72. [PubMed: 16678438]
33. Xing H, Zhang S, Weinheimer C, Kovacs A, Muslin AJ. 14-3-3 proteins block apoptosis and differentially regulate MAPK cascades. EMBO J 2000;19:349–58. [PubMed: 10654934]
34. Yeh S, Lin HK, Kang HY, et al. HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. Proc Natl Acad Sci U S A 1999;96:5458–63. [PubMed: 10318905]
35. Gregory CW, Whang YE, McCall W, et al. Heregulin-induced activation of HER2 and HER3 increases androgen receptor transactivation and CWR-R1 human recurrent prostate cancer cell growth. Clin Cancer Res 2005;11:1704–12. [PubMed: 15755991]
36. Liu Y, Majumder S, McCall W, et al. Inhibition of HER-2/neu kinase impairs androgen receptor recruitment to the androgen responsive enhancer. Cancer Res 2005;65:3404–09. [PubMed: 15833875]
37. Bakin RE, Gioeli D, Sikes RA, Bissonette EA, Weber MJ. Constitutive activation of the Ras/Mitogen-activated protein kinase signaling pathway promotes androgen hypersensitivity in LNCaP prostate cancer cells. Cancer Res 2003;63:1981–9. [PubMed: 12702592]
38. Quayle SN, Sadar MD. 14-3-3 sigma increases the transcriptional activity of the androgen receptor in the absence of androgens. Cancer Lett 2007;254:137–45. [PubMed: 17433535]
39. Tan J-A, Hall SH, Petrusz P, French FS. Thyroid receptor activator molecule, TRAM-1, is an androgen receptor coactivator. Endocrinology 2000;141:3440–50. [PubMed: 10965917]
40. Ding XF, Anderson CM, Ma H, et al. Nuclear receptor binding sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator (SRC-1): multiple motifs with different binding specificities. Mol Endocrinol 1998;12:302–13. [PubMed: 9482670]
41. McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. Endo Rev 1999;20:321–44.
42. Bin H, Wilson EM. Electrostatic modulation in steroid receptor recruitment of LXXLL and FXXLF motifs. Mol Cell Biol 2003;23:2135–50. [PubMed: 12612084]
43. Carascossa S, Gobinet J, Georget V, et al. Receptor-interacting protein 140 is a repressor of the androgen receptor activity. Mol Endocrinol 2006;20:1506–18. [PubMed: 16527872]
44. Tzivion G, Avruch J. 14-3-3 Proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. J Biol Chem 2002;277:3061–4. [PubMed: 11709560]
45. Gardino AK, Smerdon SJ, Yaffe MB. Structural determinants of 14-3-3 binding specificities and regulation of subcellular localization of 14-3-3-ligand complexes: a comparison of the X-ray crystal structures of all human 14-3-3 isoforms. Semin Cancer Biol 2006;16:173–82. [PubMed: 16678437]
46. Zhou ZX, Kemppainen JA, Wilson EM. Identification of three proline-directed phosphorylation sites in the human androgen receptor. Mol Endocrinol 1995;9:605–15. [PubMed: 7565807]
47. Gioeli D, Black BE, Gordon V, et al. Stress kinase signaling regulates androgen receptor phosphorylation, transcription, and localization. Mol Endocrinol 2006;20:503–15. [PubMed: 16282370]
48. Wong HY, Burghoorn JA, Van Leeuwen M, et al. Phosphorylation of androgen receptor isoforms. Biochem J 2004;383:267–76. [PubMed: 15239671]
49. Taneja SS, Ha S, Swenson NK, et al. Cell-specific regulation of androgen receptor phosphorylation in vivo. J Biol Chem 2005;280:40916–24. [PubMed: 16210317]

50. Haendler B, Schuttke I, Schleuning WD. Androgen receptor signalling: comparative analysis of androgen response elements and implication of heat-shock protein 90 and 14-3-3eta. Mol Cell Endocrinol 2001;173:63–73. [PubMed: 11223178]
51. Myers RB, Oelschlager DK, Manne U, Coan PN, Weiss H, Grizzle WE. Androgenic regulation of growth factor and growth factor receptor expression in the CWR22 model of prostatic adenocarcinoma. Int J Cancer 1999;82:424–9. [PubMed: 10399960]
52. Meehan KL, Sadar MD. Quantitative profiling of LNCaP prostate cancer cells using isotope-coded affinity tags and mass spectrometry. Proteomics 2004;4:1116–34. [PubMed: 15048993]
53. Huang D, Liu X, Plymate SR, et al. Proteomic identification of 14-3-3 sigma as a common component of the androgen receptor and the epidermal growth factor receptor signaling pathways of the human prostate epithelial cell line M12. Oncogene 2004;23:6881–9. [PubMed: 15300238]
54. Yano M, Nakamuta S, Wu X, Okumura Y, Kido H. A novel function of 14-3-3 protein: 14-3-3 {zeta} is a heat shock-related molecular chaperone that dissolves thermal-aggregated proteins. Mol Biol Cell. 2006 Epub ahead of print.
55. Porter GW, Khuri FR, Fu H. Dynamic 14-3-3/client protein interactions integrate survival and apoptotic pathways. Semin Cancer Biol 2006;16:193–202. [PubMed: 16697216]
56. Hermeking H, Benzinger A. 14-3-3 proteins in cell cycle regulation. Semin Cancer Biol 2006;16:183–92. [PubMed: 16697662]
57. Hoang AT, Huang J, Rudra-Ganguly N, et al. A novel association between the human heat shock transcription factor 1 (HSF1) and prostate adenocarcinoma. Am J Pathol 2000;156:857–64. [PubMed: 10702402]
58. Bin H, Bai S, Hnat AT, et al. An androgen receptor NH_2-terminal motif interacts with the COOH terminus of the Hsp70-interacting protein (CHIP). J Biol Chem 2004;279:30643–53. [PubMed: 15107424]
59. Dai Q, Zhang C, Wu Y, et al. CHIP activates HSF1 and confers protection against apoptosis and cellular stress. EMBO J 2003;22:5446–58. [PubMed: 14532117]

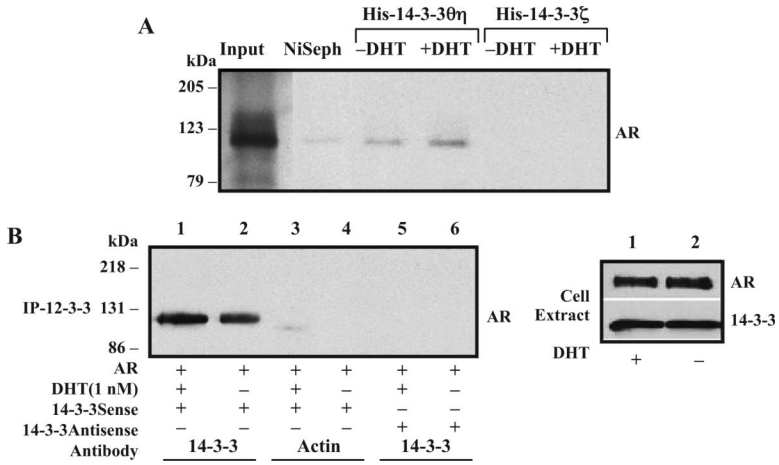

Figure 1.

Interaction between wildtype AR and 14-3-3η in vitro (A) Binding of [^{35}S] AR to His-tagged 14-3-3η in the absence or presence of 1 μM DHT was assayed in three independent experiments. AR bound 14-3-3η in the presence of DHT (*mean* & sd, NiSeph. control 9288 −/+ 981, +DHT His-tagged 14-3-3η *15032 −/+ 2119, P value = 0.013*) but not in the absence of DHT. AR did not bind His-tagged 14-3-3ζ. (B) *Left panel*: AR co-immunoprecipitation with 14-3-3η from protein lysates of COS cells. Cells were co-transfected with AR and 14-3-3η sense or antisense expression vector and incubated in the absence or presence of 1 nM DHT. Immunoprecipitates from cell lysates with anti-14-3-3 antibody, Santa Cruz Biotechnology sc-731 (lanes 1, 2, 5, 6) or anti-actin antibody (lanes 3, 4) were analysed by electrophoresis and immunoblot as described in Methods using AR monoclonal antibody (Biogenex). The positions of molecular weight markers (kDa) and migration of AR (M_r 112,000) are indicated. *Right panel*: Cell extract controls showing immunoblots of AR and 14-3-3η in protein lysates of cotransfected COS cells cultured with (lane 1) or without (lane 2) DHT. (C) *Upper panel*: COS cells were transfected with either 14-3-3η, AR or the AR corepressor CRIF1 and cultured in the presence or absence of 10 nM DHT. *Lower panel*: AR was co-transfected with 14-3-3η or CRIF1 into COS cells and cultured in the presence or absence of 10 nM DHT. AR immunofluoresence is (red) and 14-3-3η or CRIF (green). AR co-localization with 14-3-3η or CRIF is shown in merged images (yellow).

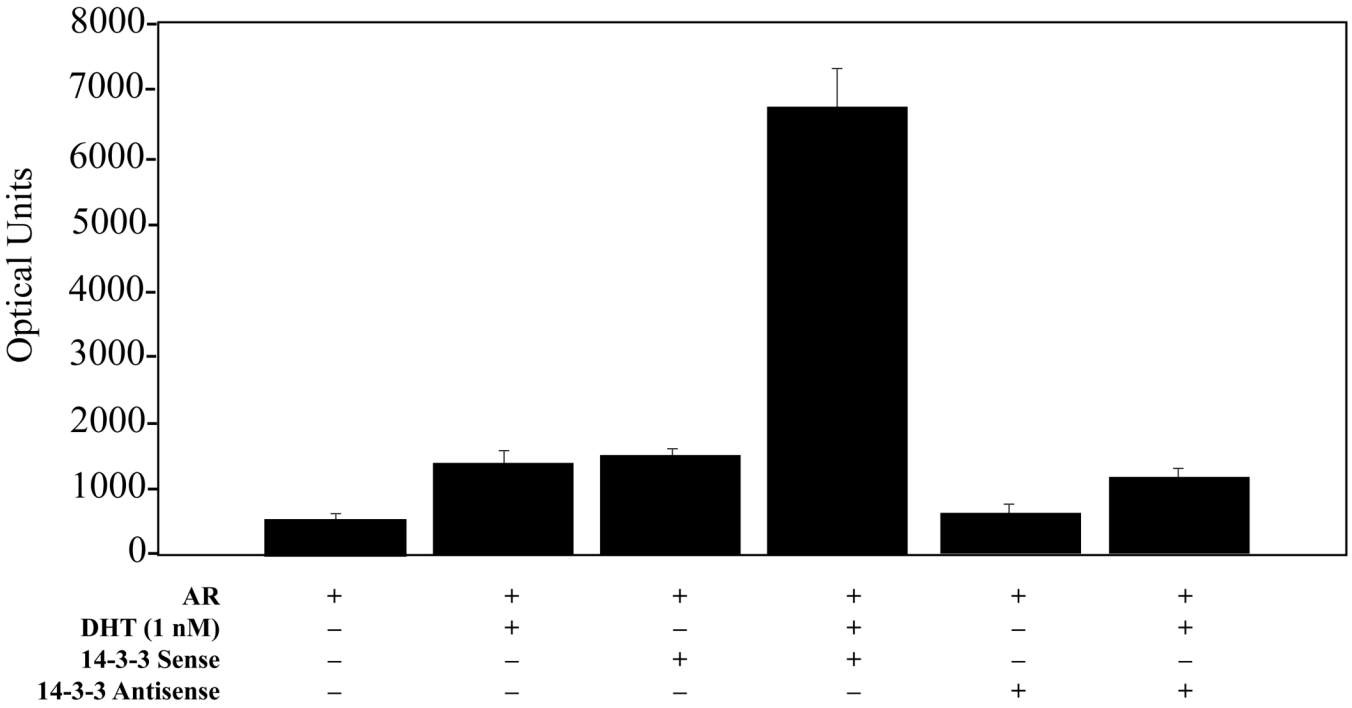

Figure 2.

(A) 14-3-3η stimulation of AR transcriptional activity. CV-1 cells in 6 cm culture dishes were cotransfected with probasin-luciferase (0.25 μg) and wild-type AR (0.25 μg) together with 14-3-3η sense or antisense vectors (0.5 μg) and incubated in the absence or presence of 1 nM DHT.

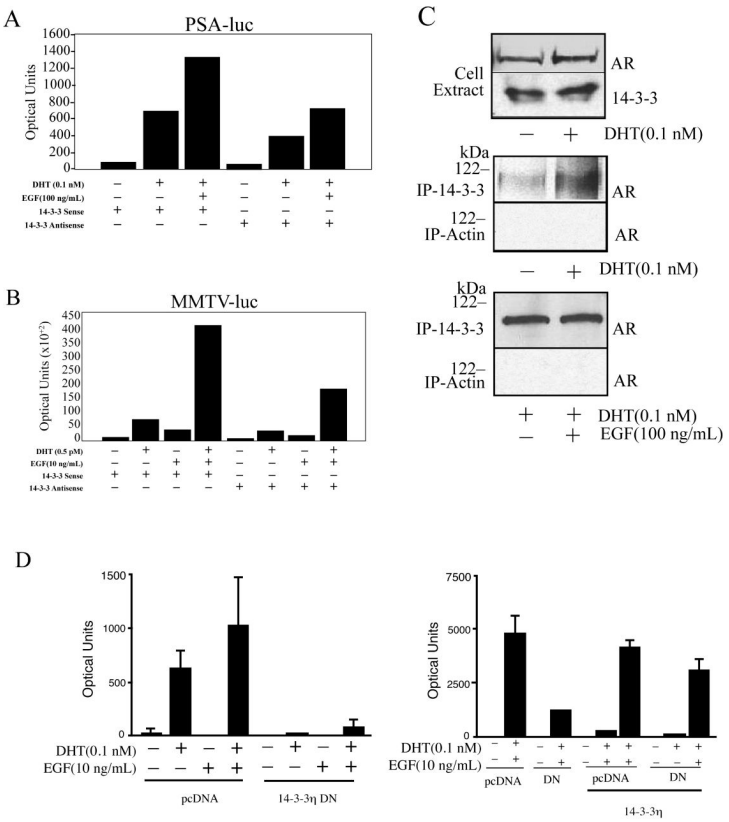

Figure 3.

14-3-3η stimulation of endogenous AR-H874Y transcriptional activity in CWR-R1 cells. CWR-R1 cells in 6 cm culture dishes were cotransfected with (A) PSA-luciferase (0.5 μg) and incubated with or without 0.1 nM DHT and 100 ng/ml EGF or (B) MMTV-luciferase (0.5 μg) with or without 0.5 pM DHT and 10 ng/ml EGF. Both A and B cotransfections included 14-3-3η sense or antisense (0.25 μg). (C) Coimmunoprecipitation of endogenous AR and 14-3-3 in protein lysates of CWR-R1 cells cultured in the absence or presence of 0.1 nM DHT, *Upper panel*: Cell protein lysates immunoblotted with AR antibody and 14-3-3η antibody sc-731. *Middle panel*: Cell extract protein lysate immunoprecipitated with anti-14-3-3η antibody sc-731 or control anti-actin antibody and immunoblotted with AR antibody. *Lower panel*: Protein lysates from CWR-R1 cells cultured in the presence of 0.1 nM DHT with or without 100ng/ml EGF were immunoprecipitated using anti-14-3-3 antibody sc-731 or control anti-actin antibody and immunoblotted with AR antibody. (D) CWR-R1 cells cultured as above in 6 cm dishes, were transfected with MMTV-luciferase (0.5 μg). *Left panel*: cells were cotransfected with either pcDNA empty vector (0.25 μg) or pcDNA expressing the dominant negative (DN)14-3-3η mutant (R 56,60 A) (0.25μg). Cells were incubated without or with DHT or EGF, or with DHT+EGF. *Right panel*: cells were transfected with either pcDNA or DN (0.125 μg) without or with pSG5-14-3-3η and incubated in the presence or absence of EGF +DHT.

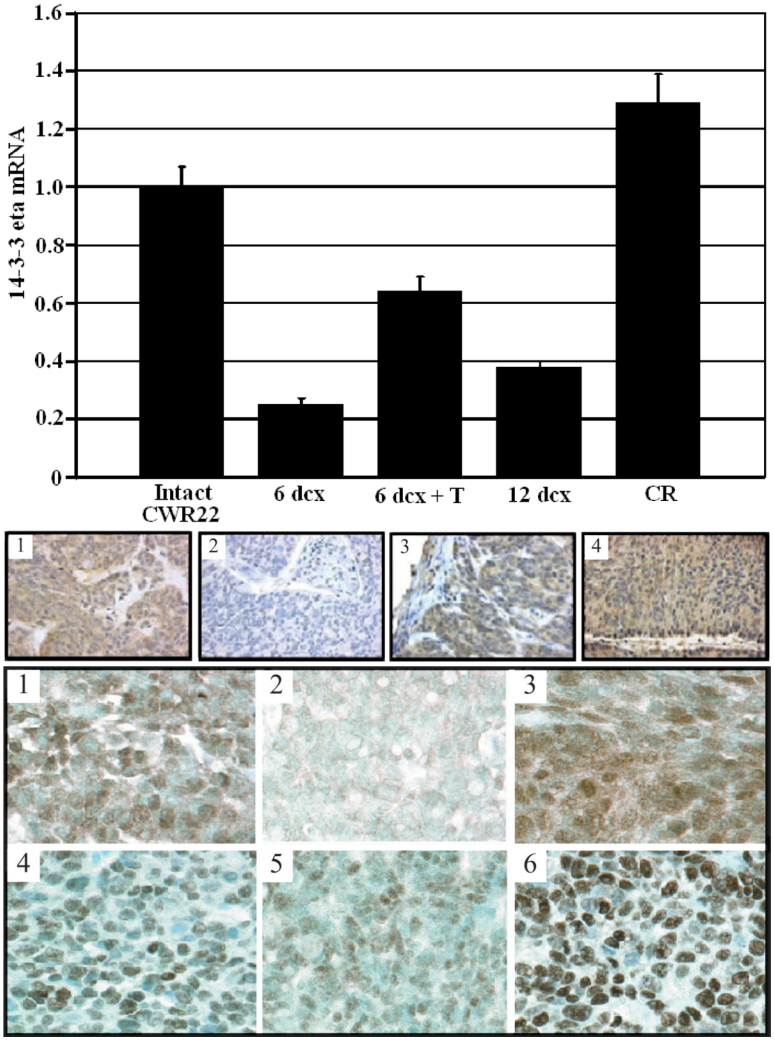

Figure 4.

Panel A: 14-3-3η mRNA assayed by quantitative RT-PCR in CWR22 xenograft tumors: intact androgen-stimulated, 6 days post-castration (6 dcx), 6 days post-castration and 48 h after injection of the host mouse with testosterone propionate 0.1 mg (6 dcx + T), 12 day post-castration untreated (12 dcx) and castration-recurrent (CR). *Panel B*: (1–4) CWR22 tumors immunostained with anti 14-3-3 antibody sc-731, *Panel C*: (1–3) CWR22 tumors stained with 14-3-3η antibody sc-17287 and (4-6) anti AR antibody. (B-1, C-1, C-4) CWR22 tumor from intact mouse (B-2, C-2, C-5) CWR22 tumor from mouse 6 days post-castration (B-3) CWR22 tumor 6 days post-castration and 48 h after injection of mouse with testosterone propionate 0.1 mg, (B-4, C-3, C-6) castration-recurrent CWR22 tumor from mouse 150 days post-castration. Images in panel B were reduced from X100 and images in panel C obtained with a X40 lens using the SPOT-4 camera system were reduced from X400.

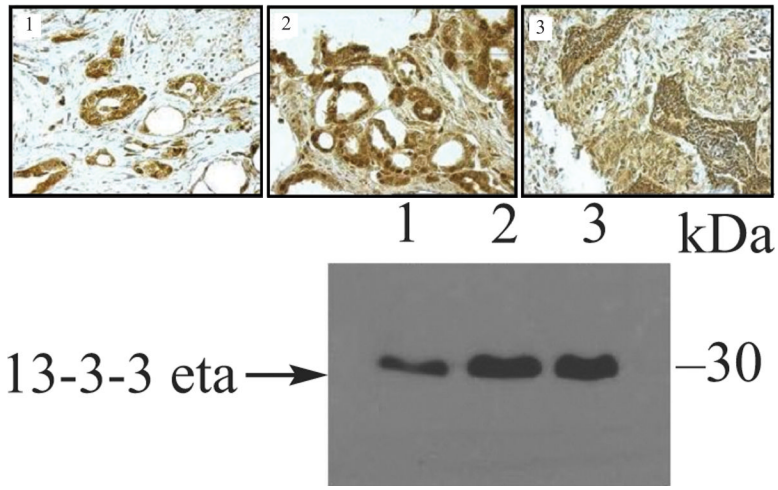

Figure 5.

14-3-3η immunostaining with sc-17287 in human prostate tissue microarray sections of clinical specimens (27) (A) from: 1) benign prostate, untreated 2) prostate cancer, untreated 3) castration-recurrent prostate cancer. All original images were reduced from X400. (B) 14-3-3η immunoblot using sc-17287 antibody and protein extracts prepared from random samples of (1) benign prostate, untreated (2) prostate cancer, untreated (3) castration-recurrent prostate cancer.

Table 1

Nuclear and cytoplasmic visual scores of 14-3-3η and androgen receptor immunostaining in tissue microarrays comprised of androgen-stimulated benign prostate, androgen-stimulated prostate cancer and castration-recurrent prostate cancer (mean ± SEM).

		Androgen-stimulated benign prostate	Androgen-stimulated prostate cancer	Castration-recurrent prostate cancer
14-3-3η	Nuclear	255 ± 11	247 ± 19	256 ± 10
	Cytoplasmic	246 ± 12	212 ± 25	255 ± 11
Androgen Receptor	Nuclear	212 ± 5	225 ± 6	220 ± 6
	Cytoplasmic	0	0	0