Role of SRC-1 in the Promotion of Prostate Cancer Cell Growth and Tumor Progression


Departments of 1Molecular and Cellular Biology, 2Pathology, and 3Urology, Baylor College of Medicine, Houston, Texas

Abstract

Prostate cancer is initially androgen dependent and there is evidence that androgen receptor continues to play a role in androgen-independent prostate cancer. Androgen receptor activity depends both on the level of androgens and on the level of coactivators that interact with androgen receptor. Our goal was to evaluate the role of the androgen receptor coactivator SRC-1 in prostate cancer progression. Using tissue arrays to measure SRC-1 protein levels, we found that increased SRC-1 expression in clinically localized, androgen-dependent cancer is associated with clinical and pathologic variables of increased tumor aggressiveness. Interestingly, there was variable expression of SRC-1 in normal prostate tissue which correlated with the staining intensity of the corresponding cancer tissue. To test the contribution of SRC-1, we examined its role in androgen-dependent LNCaP and androgen-independent C4-2 prostate cancer cell lines. Using small interfering RNA to reduce expression of androgen receptor, we found that androgen receptor was required both for cell growth and for basal expression of prostate-specific antigen in the androgen-independent C4-2 cell line. Thus, although the cells can grow in an androgen-depleted medium, they remained androgen receptor dependent. Reduction of SRC-1 expression significantly reduced growth and altered androgen receptor target gene regulation in both LNCaP and C4-2 cell lines whereas it had no effect on the growth of the androgen receptor–negative PC-3 and DU145 prostate cancer cell lines. Although the requirement for androgens and androgen receptor in the development of prostate cancer is well established, our study implicates enhanced androgen receptor activity through elevated expression of SRC-1 in the development of more aggressive disease in men with prostate cancer. (Cancer Res 2005; 65(17): 7959-67)

Introduction

Prostate cancer has the highest incidence and second highest mortality of all cancers in U.S. men. Because prostate cancer is initially androgen dependent, androgen ablation is the treatment of choice for metastatic disease. Current means of blocking androgen action are insufficient to cure the disease and androgen-independent tumors often develop. The actions of androgens are mediated by the androgen receptor, a member of the nuclear receptor family of ligand-activated transcription factors (1). On binding hormone, androgen receptor binds to androgen receptor response elements in androgen receptor–responsive promoters, recruits multiple coactivators, and activates transcription (2, 3). The activity of androgen receptor depends not only on the concentration of hormone and receptor but also on the milieu of coactivators and the activity of cell signaling pathways that phosphorylate androgen receptor and its coactivators. Consequently, overexpression or hyperactivation of components of the androgen receptor signaling pathway may contribute to accelerated androgen-dependent prostate cell growth. Although many candidate coactivators have been identified (3), the contribution of the individual proteins to androgen receptor action in normal prostate or prostate cancer is unknown. A limited study suggested that SRC-1 is overexpressed in androgen-independent prostate cancer as compared with benign prostatic hyperplasia or androgen-dependent cancer (4). Thus, we sought to determine whether SRC-1, which is required for optimal androgen-dependent prostate growth in mice (5), is overexpressed in androgen-dependent prostate cancer. We analyzed expression of SRC-1 protein in normal prostate and prostate cancer tissues using tissue microarrays and found that increased expression of SRC-1 directly correlates with the aggressive behavior of the clinically localized, androgen-dependent prostate cancer.

To evaluate the role of SRC-1 in androgen-dependent prostate cancer cell growth and gene expression, we reduced expression of SRC-1 in androgen-dependent LNCaP cells and found that SRC-1 is necessary both for growth and for androgen receptor target gene regulation. Although our primary goal was to assess the expression and role of SRC-1 in androgen-dependent prostate cancer, there is an abundance of recent evidence implicating androgen receptor action in the growth of androgen-independent prostate cancers. Androgen receptor is often overexpressed (6) in androgen-independent tumors as are its primary target genes such as prostate-specific antigen (PSA). Recent reports have shown that androgen receptor is reactivated independent of testicular androgens in a prostate cancer xenograft model when the tumors become hormone refractory (7) and that overexpression of androgen receptor is the single most consistent event during transition to androgen independency (8). We show here that C4-2 cells, an androgen-independent derivative of LNCaP cells, which grow in castrated mice and can also grow in charcoal-stripped serum lacking significant levels of androgen, remain androgen receptor dependent and that SRC-1 is required for growth and androgen receptor–dependent gene expression in androgen-depleted medium. Thus, reducing the expression or functional interaction between SRC-1 and androgen receptor represents a new therapeutic target for both androgen-dependent and androgen-independent (but androgen receptor dependent) prostate cancer.

Materials and Methods

Cell cultures. LNCaP, HeLa, DU145, and PC-3 cells were obtained from the American Type Culture Collection (Manassas, VA). LNCaP cells were
maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Intergen Co., Purchase, NY) with penicillin and streptomycin (Invitrogen). Hela cells were grown in DMEM (Invitrogen) with 10% FBS and penicillin/streptomycin. PC-3 cells were maintained in DMEM/F12 with 10% FBS and penicillin/streptomycin. DU145 cells were grown in MEM + 1% 1-glutamine with 10% FBS. The C-2 cell line was purchased from UroCox, Inc. (Oklahoma City, OK) and propagated in T medium with 5% FBS (9). All cell lines were maintained at 37°C in a humid atmosphere containing 5% CO2.

Materials. [3H]Thymidine was purchased from ICN (Irvine, CA). Rabbit anti-mouse immunoglobulin G (IgG) was obtained from Zymed Laboratories, Inc. (San Francisco, CA). Horseradish peroxidase–conjugated anti-rabbit IgG and enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). R1881 (methyltrienolone) was purchased from Perkin-Elmer (Boston, MA). Tissue culture disposable supplies were obtained from Fisher Scientific (Pittsburgh, PA). All chemicals were reagent grade unless otherwise indicated. GREGel-1b-Luc, pcRS1.1AR, pcRS1.1Gal, and pcRS1SRC-1 were previously described (10, 11). Mutated androgen receptor TR877A was obtained from Dr. Steven Balk (Dana-Farber Cancer Institute, Boston, MA; ref. 12). The expression plasmid was digested with BspHI restriction enzyme, the ends were blunted, and the plasmid was then digested with Nhel. The 2.952 bp fragment containing the androgen receptor sequence was purified and cloned into the Nhel-FmeI sites of the pc83.1 expression vector (Invitrogen).

RNA interference. C4-2 cells were grown to 40% confluence in six-well plates in RPMI media supplemented with 5% charcoal-stripped FBS. Two hundred picomoles of either androgen receptor–specific or control small interfering RNA (siRNA) duplexes were used for transfection. Two hundred picomoles of either androgen receptor–specific or control small interfering RNA (siRNA) duplexes per well were incubated with 10 μL of Lipofectamine reagent (Invitrogen) as recommended in the protocol of the manufacturer, added to the cells in serum-free medium, and incubated for 6 hours. Equal volumes of medium containing 10% charcoal-stripped FBS were added to bring the final concentration of serum to 5%. After an additional 24 or 48 hours, growth assays, protein analyses, RNA extractions, and immunofluorescence studies were done. The androgen receptor–specific siRNA construct sequence was (r)GACCUCGGAGGAGCUUUCdTdT and a strand complementary to that with dTdT on the 3′ end. The control sequence had the same composition, except the positions of two nucleotides were exchanged (underlined): r(GACCUCGGAGGAGCUUUC)dtdT and a strand complementary to that with dTdT on the 3′ end. The androgen receptor–specific siRNA used was a smart pool (Dharmacon, Lafayette, CO), which was used with noncoding siRNA control 1 (Ambion, Austin, TX). The double-stranded siRNAs were purchased from Dharmacon. SRC-1 siRNA and control 1 were used exactly as designed as gapmers with 2′ methyl-deoxynucleotides in the middle to promote RNase H activity.

Western blot analysis. Transfected cells were lysed with TESH lysis buffer [10 mmol/L Tris-HCl (pH 7.7), 1 mmol/L EDTA, 12 mmol/L thiglycolerol] and 10 to 15 μg of protein were resolved on 12.5% SDS-PAGE. After transfer, proteins were detecting using either a PSA antibody (12,000 dilution; DAKO, Carpinteria, CA) or an actin antibody (1:1,000 dilution; Chemicon International, Temecula, CA) and an ECL (Amersham) detection kit. For androgen receptor detection, 15 to 25 μg of protein lysate were run on 6.5% SDS-PAGE and androgen receptor was detected with AR441 antibody as previously described (11).

Preparation of RNA from cells and real-time quantitative PCR. Total RNA was extracted using TRizol reagent (Invitrogen) as recommended by the manufacturer and dissolved in 60 μL of diethylpyrocarbonate-treated water. As a template, 2 to 10 μL of RNA solution were used for real-time quantitative reverse transcription-PCR (RT-PCR) of SRC-1, androgen receptor, and maspin mRNA using TaqMan One-Step RT-PCR master mix reagents and an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). All data were normalized to 18S rRNA expression. To measure 18S expression, the primary RNA stock was diluted 1,000-fold and 5 μL were used in a RT-PCR reaction using TaqMan RNA Control and One-Step RT-PCR Master Mix Reagents (Applied Biosystems). Each point was done in triplicate and the average and SD were calculated. Maspin was detected using the following primers and TaqMan probe: CAGATGGCCACCTTTGAGACATC, GCCAGCATTAACCCACAGGATT, and 6FAM-CAATTCCGAAAAGCCCTATTCCCA-TAMRA. For SRC-1, the primers and probe were TGAAGTGGAAGAAAGAACACAGATG, GTCAAGGTCAGCTGTAACCTGG, and 6FAM-CAACAAAATCTCTCGAGGAAATAAAA-CTTGGAG-TAMRA. For androgen receptor, primers and probe were GCCGCCATGCTCGTTTTTCA, CATTGCCAGACACTGCGTGT, and 6FAM-AGTCGCGATCGACAGTCCG-GGA-TAMRA (Applied Biosystems).

Immunofluorescence. C4-2, LNCaP, and PC-3 cells were grown on coverslips in six-well plates in medium with full serum for LNCaP and PC-3, or with charcoal-stripped serum for C4-2. For detection of androgen receptor, C4-2 cells were fixed in 4% formaldehyde and incubated with AR441 antibody and goat anti-mouse antibody and FITC–conjugated goat anti-mouse immunoglobulin M (Zymed) as previously described (16). For SRC-1, we used the same protocol except the primary antibody for SRC-1, M-341 (Santa Cruz Biotechnology, Santa Cruz, CA), was used at 1:1,000 dilution. The fluorescent signal was detected using a Zeiss AxioPlan2 microscope. The optimal exposure was found for detection of the fluorescent signal in control siRNA– or oligonucleotide-treated cells. Fluorescence in cells treated with androgen receptor–specific siRNA or SRC-1 antisense oligonucleotides was captured with the same exposure times as controls and left unscaled using MetaView software.

Tissue microarrays and immunohistochemistry. The tissue microarrays used to study SRC-1 expression in 516 clinically localized prostate cancer have been previously described (17). Radical prostatectomy patients received no adjuvant therapy such as radiation or hormonal therapy. Other patient characteristics were as previously described (17). For the metastatic tissue microarray, 2 mm tissue cores were obtained from a cohort of 177 cases of metastatic prostate cancer. These included metastases in lymph node (162), bone (9), liver (1), brain (1), lung (1), paraspinal region (1), lymphus (1), and spinal cord (1). Of these, 139 patients received no hormonal therapy before biopsy or surgery. Four had received neoadjuvant hormonal therapy. The status of the remaining cases is unknown. Of these, 91 cases were evaluable. Immunohistochemistry was done as previously described (18). Antigen retrieval was done in 10 mmol/L citrate buffer (pH 6.0) for 30 minutes in a rice cooker. Endogenous biotin and peroxidase were blocked using appropriate kits from Vector Laboratories (Burlingame, CA) according to the protocol of the manufacturer. Anti-SRC-1 mouse

Transactivation assay. The indicated DNAs were transfected using poly-c-laccine–conjugated adenovirus as previously described (15). Cells were lysed 24 hours later and assayed for luciferase and β-Gal activity (11).

Growth assay. Cells were transfected with either siRNA duplexes or oligonucleotides and, at the indicated times after transfection, [3H]thymidine was added to the medium to a final concentration of 2 μCi/mL. Cells were incubated for another 2 hours, fixed, and incorporated [3H]thymidine was determined as previously described (14).

Western blot analysis. Transfected cells were lysed with TESH lysis buffer [10 mmol/L Tris-HCl (pH 7.7), 1 mmol/L EDTA, 12 mmol/L thiglycolerol] and 10 to 15 μg of protein were resolved on 12.5% SDS-PAGE. After transfer, proteins were detecting using either a PSA antibody (12,000 dilution; DAKO, Carpinteria, CA) or an actin antibody (1:1,000 dilution; Chemicon International, Temecula, CA) and an ECL (Amersham) detection kit. For androgen receptor detection, 15 to 25 μg of protein lysate were run on 6.5% SDS-PAGE and androgen receptor was detected with AR441 antibody as previously described (11).
monoclonal antibody (MAI-840, Affinity Bioreagents, Golden, CO) was incubated with each tissue array section at 300 ng/mL at 4°C overnight followed by the avidin-biotin peroxidase complex procedure (Vector Laboratories) and counterstaining with hematoxylin as previously described (18). Slides were scanned using a Bliss automated slide scanner system to produce high-resolution digital images. Staining was evaluated in the epithelial nuclei in normal luminal epithelium and prostate cancer as previously described (17). Staining intensity was graded as absent (0), weak (1+), intermediate (2+), or strong (3+). The extent of staining was estimated and scored as follows: no staining (0), 1% to 33% of cells stained (1+), 34% to 66% of cells stained (2+), or 67% to 100% of cells stained (3+). The staining index for each case was then calculated by multiplying the average intensity score for the three cores by the average percentage score for the three cores, yielding a 10-point tumor staining index ranging from 0 (no staining) to 9 (extensive, strong staining) for each case.

Results

Analysis of SRC-1 expression in prostate and prostate tumors. To evaluate expression of SRC-1 in clinically localized human prostate cancers, we did immunohistochemistry on tissue microarrays using an anti–SRC-1 antibody. Tissue arrays were constructed from radical prostatectomy specimens using a uniform protocol developed by the Baylor Prostate Cancer Specialized Program of Research Excellence. Stained slides were digitized and scored both for extent of staining (scale of 0-3) and intensity of staining (scale of 0-3; see Materials and Methods). A staining index was calculated from the average extent of staining score for the three cores multiplied by the average staining intensity score so that the staining index ranged from 0 (no staining) to 9 (extensive, strong staining). Staining was present primarily in basal and luminal epithelial cell nuclei with staining of some stromal cell nuclei. For normal epithelium, only luminal epithelial cells were scored. Interestingly, the normal epithelium had quite variable staining indices, ranging from 0 to 9. The three cores from each patient were highly correlated with each other so that this variability seemed to reflect differences between patients rather than heterogeneity within specimens. Examples of the variable staining of normal tissues are shown in Fig. 1A (a–c).

Figure 1. Analysis of SRC-1 expression in prostate. A, expression of SRC-1 protein in normal prostate (a-c) and prostate cancer (d–h) was determined using tissue microarrays and staining was scored as described in Materials and Methods. a, normal prostate peripheral zone tissue with no expression of SRC-1 (staining index, 0). b, normal prostate with intermediate expression of SRC-1 in the luminal epithelium (staining index, 0). c, normal prostate with strong SRC-1 staining (staining index, 9). d, prostate cancer with no SRC-1 expression. The normal epithelium is also negative. e, prostate cancer with low SRC-1 expression (staining index, 2). f to h, prostate cancers with strong SRC-1 expression (staining index, 9). Note strong staining of normal luminal epithelium in F (arrow). B, Spearman’s nonparametric correlations of src-1 tumor staining index and clinical-pathological variables associated with prostate cancer progression. The tumor staining index was determined as described in Materials and Methods following immunohistochemical staining of tissue microarrays containing triplicate samples from 516 prostate cancers and corresponding normal tissue using an anti–SRC-1 antibody.
again this seemed to represent variation between patients rather than heterogeneity within a given tumor. Examples of tumor staining are shown in Fig. 1A. Twenty-four hours posttransfection, cells were fixed, incubated with androgen receptor primary antibody and FITC-conjugated rabbit anti-mouse secondary antibody, and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). D, cells were transfected with either control or androgen receptor–specific siRNA, harvested at the indicated time points, and 15 μg of protein extract were analyzed for PSA and actin expression by Western blotting. E, cells were transfected in parallel with B and, at the indicated time points, RNA was prepared and analyzed for androgen receptor mRNA and 18S RNA expression. Androgen receptor expression was then normalized using 18S levels, and expression in the control wells at 12 hours was designated as 100. Each data point was done in triplicate. F, C4-2 cells were plated in six-well plates and transfected as in A and, at the indicated time points, cell growth was evaluated by [3H]thymidine incorporation. In this and all subsequent figures, each sample was done in triplicate, each experiment was done at least thrice, and a representative experiment is shown. G, C4-2 cells were transfected with androgen receptor–specific smart pool siRNA (si2AR) or nontargeting control siRNA (C#1) as in B and, at 24 and 48 hours, cells were harvested and RNA was extracted and analyzed for androgen receptor expression as in E. H, C4-2 cells were transfected in parallel with H and analyzed for growth by [3H]thymidine incorporation.
SRC-1 staining in the metastatic lesions compared with the clinically localized prostate cancers (6.286 versus 5.42; $P = 0.005$, Wilcoxon rank-sum test). Thus, enhanced SRC-1 expression is most likely associated with fatal disease progression.

**C4-2 cells are an androgen-independent, but androgen receptor–dependent, prostate cancer cell line.** The correlation between SRC-1 expression and poor prognosis led us to test the contribution of SRC-1 to cell growth using prostate cancer cell lines including the androgen-dependent LNCaP cells and its androgen-independent derivative C4-2 (19). There is evidence that some prostate tumors that grow in the absence of normal circulating levels of androgens remain androgen receptor dependent (4, 20); alterations in androgen receptor and/or cofactor expression or cellular signaling may allow androgen receptor to function under these conditions (4, 7, 8, 21, 22). In contrast to LNCaP cells, C4-2 cells grow in castrate mice and grow in vitro in charcoal-stripped serum depleted of androgens. Under these conditions, PSA expression in LNCaP cells depends on added androgen (Fig. 2A), whereas C4-2 cells express PSA without added androgens (Fig. 2B, controls). These findings raised the question of whether the androgen-independent C4-2 cells remain dependent on androgen receptor. Therefore, we treated C4-2 cells grown in charcoal-stripped serum with either control or androgen receptor–specific siRNA. Cells treated with androgen receptor–specific siRNA have markedly lower androgen receptor protein and mRNA levels (Fig. 2C) compared with those treated with the control siRNA. Remarkably, in media containing charcoal-stripped serum, the decrease in androgen receptor levels reduced both [3H]thymidine incorporation (a surrogate for cell growth; Fig. 2D) and PSA expression in LNCaP cells (Fig. 2E). Similar results were obtained with smart pool siRNA for androgen receptor (siAR) and nontargeting control siRNA C1 (Fig. 2G and H and data not shown). Therefore, in androgen-independent C4-2 cells, androgen receptor is required both for cell growth and PSA expression despite the lack of androgen in the medium. Thus, these cells serve as a model to test the role of SRC-1 in androgen-independent, but androgen receptor–dependent, prostate cancer.

**SRC-1 preferentially potentiates the activity of androgen receptor at suboptimal levels of hormone.** Androgen receptor in LNCaP cells contains a T877A substitution which broadens ligand specificity (23). To confirm that SRC-1 also coactivates this mutant androgen receptor, PC-3 cells were cotransfected with expression vectors for SRC-1 and androgen receptor and transcriptional activity was measured; as predicted, SRC-1 stimulates the activity of the T877A mutant (Fig. 3A). To test whether SRC-1 can potentiate the minimal activity of androgen receptor at very low levels of hormone such as those remaining following androgen ablation, transfected HeLa cells were treated with 1 nmol/L or 5 pmol/L R1881 and the luciferase activity was measured. SRC-1 not only enhances the androgen receptor activity at 5 pmol/L R1881 (Fig. 3B) but actually causes a greater fold induction of activity (see inset) than at 1 nmol/L R1881. Thus, SRC-1 has the capability to enhance androgen receptor activity both at optimal and suboptimal levels of hormone. To assess the contribution of SRC-1 in prostate cancer cells, we measured cell growth, a positively regulated androgen receptor target (PSA), and a negatively regulated androgen receptor target (maspin; ref. 24). SRC-1 mRNA (Fig. 3C) is expressed in all four cell lines, although at slightly lower levels in androgen receptor–negative PC-3 and DU145 cells. Androgen receptor mRNA is expressed at similar levels in C4-2 and LNCaP cell lines, but not in PC-3 or DU145 cells, as expected (Fig. 3D). Maspin mRNA, a gene negatively regulated by androgen receptor (24), is also expressed in LNCaP and C4-2 cell lines, but is much higher in the PC-3 and DU145 cell lines, which lack androgen receptor (Fig. 3E).

![Figure 3. SRC-1 preferentially potentiates the activity of androgen receptor at low hormone levels.](image-url)

**Figure 3. SRC-1 preferentially potentiates the activity of androgen receptor at low hormone levels.** A, PC-3 cells were transfected with 400 ng of GRE,E1b-Luc reporter, 4 ng of pCR3.1AR wild-type or T877A mutant, and 400 ng of either pCR3.1SRC-1 or pCR3.1 vector. Cells were treated with 3 nmol/L R1881 or left untreated. Cellular lysates were analyzed for luciferase activity 24 hours after transfection. Each point was done in triplicate and luciferase values were normalized for ß-Gal expression. Bars, SD. B, HeLa cells plated in six-well plates were transfected with 5 ng pCR3.1AR, 400 ng GRE,E1b-Luc, and 500 ng of either pCR3.1SRC-1 or pCR3.1. After transfection, cells were treated with 1 nmol/L or 5 pmol/L R1881 or left untreated. After 24 hours of treatment, cells were lysed and assayed for ß-Gal and luciferase activity. Each data point was done in triplicate. C to E, cells were plated in 10 cm plates and grown as described in Materials and Methods. Total RNA was prepared using TRIzol (Invitrogen) and analyzed for SRC-1 (C), androgen receptor (D), and maspin (E) mRNA expression by real-time RT-PCR. Gene expression was normalized to the levels of 18S RNA.
Treatment of LNCaP, C4-2, DU145, and PC-3 cell lines with SRC-1 antisense oligonucleotide reduces levels of SRC-1 mRNA and protein. To validate the use of coactivator specific antisense oligonucleotides, we transfected cells with SRC-1–specific (antisense oligonucleotides) or control oligonucleotides and monitored mRNA levels by quantitative RT-PCR and protein levels by immunofluorescence. LNCaP, C4-2, DU145, and PC-3 cells treated with antisense oligonucleotides had significantly lower levels of SRC-1 protein (Fig. 4A) and mRNA (Fig. 4B) compared with the cells treated with the control oligonucleotides.

SRC-1 is required for optimal regulation of androgen receptor target genes and growth of LNCaP cells. LNCaP cells were treated with SRC-1 control oligonucleotides or antisense oligonucleotides resulting in an antisense oligonucleotide–dependent reduction of SRC-1 mRNA of ~60% at 48 hours (Fig. 5A, left). This treatment caused an equivalent reduction in cell growth measured by [3H]thymidine incorporation (Fig. 5B, left), supporting the hypothesis that SRC-1 activity is required for optimal cell growth. Analysis of androgen-regulated genes showed that PSA was substantially reduced (Fig. 5C). Interestingly, maspin expression was increased as a result of SRC-1 reduction (Fig. 5D). To our knowledge, this is the first report of an androgen receptor coactivator playing a role in repression of a target gene by androgen receptor. Similar effects on growth, as well as a decrease in PSA expression (data not shown), were obtained in cells treated with SRC-1–specific siRNA and a noncoding control (Fig. 5A and B, right).

SRC-1 is necessary for androgen receptor activity and growth of C4-2 cells in androgen-depleted medium. To address the contribution of SRC-1 in androgen-independent growth and gene expression, C4-2 cells in charcoal-stripped serum were treated with SRC-1 antisense oligonucleotides or control oligonucleotides. The reduction in SRC-1 expression (Fig. 5E, left) was more effective than in LNCaP cells, and the corresponding reductions in growth (Fig. 5F, left) and PSA (Fig. 5H) were also greater. Maspin expression was also increased (Fig. 5G). Similar results were obtained using control and SRC-1–specific siRNA (Fig. 5E and F, right). Thus, SRC-1 is a major contributor to androgen-independent actions of androgen receptor.

SRC-1 does not play a role in the growth of androgen receptor–negative PC-3 and DU145 prostate cancer cells. Although SRC-1 was originally identified as a nuclear receptor coactivator (25), it also serves as a coactivator for some other transcription factors. To determine whether the effects on cell growth are due to androgen receptor–mediated action or are independent of androgen receptor, expression of SRC-1 was reduced in androgen receptor–negative PC-3 cells. Strikingly, SRC-1 reduction (Fig. 6A) had no effect on PC-3 cell growth (Fig. 6B) or maspin expression (Fig. 6C). To test if this is a PC-3 cell–specific phenomenon, we conducted the same set of experiments with the DU145 cell line. Similar to PC-3 cells, reduction in SRC-1 levels (Fig. 6D) did not affect DU145 cell growth (Fig. 6E) or maspin expression (Fig. 6F). Similarly, no effect on growth was observed when SRC-1 expression was ablated with siRNA in PC-3 cells (data not shown).

Discussion

Because tumor growth is androgen dependent, androgen ablation therapy is used to treat metastatic prostate cancer. Often, the treatment is insufficient to eradicate the tumor before the emergence of tumor cells that can grow in the absence of normal levels of androgens. Androgen receptor activity is dependent on hormone and coactivator levels and on activation of cell signaling pathways. Although there are many candidate androgen receptor coactivators (3), for the most part their contribution to androgen receptor action is unknown. We studied SRC-1 because its activity is required for normal androgen-regulated prostate growth in mice (5), and its activity is positively regulated by p42/p44 mitogen-activated protein kinases (26, 27), whose activities are elevated in prostate cancer (21). Moreover, in a small study of SRC-1 expression in eight samples from benign prostate hyperplasia, eight early, and eight recurrent prostate cancer specimens, SRC-1 was expressed at higher levels in androgen-independent tumors (4), suggesting that there may be a relationship between SRC-1 expression and recurrent prostate cancer. Our large-scale study shows that SRC-1 expression correlates with several variables of more aggressive disease in primary tumors and that metastatic...
prostate cancer tissues express higher levels of SRC-1 than do primary tumors and normal prostate. Our finding that SRC-1 is overexpressed in aggressive prostate cancers as well as Debes et al.'s study (22) report on increased expression of the coactivator p300 (which is recruited to androgen receptor by SRC-1; ref. 28) in aggressive prostate cancers support a role for overexpression/increased activity of coregulators.

SRC-1 expression was extremely variable in both normal and tumor samples, although there was much less heterogeneity within samples from the same patient. An unexpected finding was the remarkable correlation between levels in normal tissue and tumor tissue from the same patient \((P < 0.00001, \rho = 0.50\), Spearman's correlation). Taken together with the correlations with aggressiveness, these data indicate that high levels of SRC-1 in normal tissue increase the risk for aggressive disease in men who develop prostate cancer. However, it is unclear why there is a correlation between SRC-1 levels in normal peripheral zone tissue and its corresponding cancer. It is possible that this reflects germ line genetic heterogeneity between different men in factors that directly or indirectly influence SRC-1 expression. Alternatively, this could represent a "field effect" in which there are widespread alterations of gene expression in apparently normal tissue in men with

**Figure 5.** SRC-1 is necessary for optimal androgen receptor activity. **A,** LNCaP cells were transfected with 400 nmol/L of SRC-1 control oligonucleotides or antisense oligonucleotides (left) and 100 nmol/L of control (C) or SRC-1–specific siRNA (right). Total RNA was isolated at the indicated times and analyzed for SRC-1 expression and normalized for 18S expression. **B,** cells were transfected in parallel with **A** and cell growth was assessed by \(^{3}H\)thymidine incorporation. **C,** LNCaP cells transfected with control and antisense oligonucleotides in parallel with **A** were harvested at the indicated time points and protein was extracted and analyzed for PSA and actin expression by Western blotting. **D,** C4-2 cells were plated in six-well plates in medium supplemented with 5% charcoal-stripped serum and transfected with 400 nmol/L of SRC-1 control oligonucleotides or antisense oligonucleotides (left) and 200 nmol/L of control 1 (Ambion) or SRC-1–specific siRNA (Ambion; right). At the indicated time points, total RNA was extracted and analyzed for SRC-1 and 18S expression. SRC-1 expression was normalized for 18S expression. Normalized expression of SRC-1 in cells treated with control oligonucleotides harvested at the earliest time point was defined as 100 and all other numbers adjusted accordingly. **E,** C4-2 cells were plated and transfected in parallel with **E** with either ISIS oligonucleotides or the indicated siRNA and, at the indicated time points, \(^{3}H\)thymidine was added at a concentration of 2 \(\mu\)Ci/mL. After 90 minutes, cells were fixed and the amount of incorporated \(^{3}H\)thymidine was determined. **F,** C4-2 cells were transfected with control and antisense oligonucleotides harvested at the earliest time point was defined as 100 and all other numbers adjusted accordingly. **G,** total RNA from C4-2 cells transfected with oligonucleotides was analyzed for maspin expression by quantitative real-time RT-PCR. Maspin expression was normalized for 18S expression; the expression in cells treated with control oligonucleotides at the 24 hour time point was assigned a value of 100. **A, B, D, E, F, and H,** each point was done in triplicate and SD was calculated.
effect in prostate cancer. A better understanding of the genetic and epigenetic factors controlling SRC-1 expression is needed to understand the basis for variable expression of SRC-1 in normal prostate tissue and prostate cancers.

When prostate cancers become refractory to androgen ablation, they typically continue to secrete PSA (the product of an androgen-regulated gene), raising the question of whether the tumor has developed an alternate means of activating the androgen receptor and, if so, how androgen receptor signaling can be inhibited. Proposed mechanisms for emergence of androgen resistance include androgen receptor mutations that alter specificity (12), activation of cell signaling (21, 30), androgen receptor overexpression (6), and coactivator overexpression (4, 22, 31). There is good evidence that androgen receptor is reactivated when tumor xenograft growth resumes in mice with castrate levels of androgens (7). In addition, injection of an androgen receptor antibody into androgen-refractory C4 cells grown in androgen-containing medium inhibited the growth of the cells (32). Our study using androgen receptor siRNA to reduce levels of androgen receptor shows that both the basal expression of PSA and the growth of androgen-independent C4-2 cells in androgen-depleted medium are androgen receptor dependent. Thus, there is evidence for a continued role of androgen receptor in androgen-independent prostate cancer. Remarkably, even a partial reduction in SRC-1 levels leads to reduced growth in both androgen-dependent and androgen-independent (but androgen receptor expressing) models, as well as to decreased PSA expression and a reversal of maspin repression. SRC-1 ablation in androgen receptor–negative PC-3 and DU145 cells had no effect on growth or maspin expression. Thus, the growth inhibition as a result of SRC-1 ablation in LNCaP and C4-2 cells seems to be dependent on androgen receptor expression.

In summary, these data suggest that SRC-1, through its interactions with androgen receptor, is a factor in prostate cancer progression. Although the requirement for androgens and androgen receptor in the development of prostate cancer is well established, our study implicates enhanced androgen receptor activity through elevated expression of SRC-1 in the development of more aggressive disease in men with prostate cancer. In contrast to other steroid receptors, which interact with SRC-1 through the LXXLL motifs in SRC-1, the strongest interactions with androgen receptor occur between the NH2 terminus of androgen receptor and a glutamine-rich region in the p160 coactivator (33). This interface represents a unique therapeutic target for both androgen-dependent and -independent, but androgen receptor–dependent, prostate cancer.

Acknowledgments

Received 10/1/2004; revised 3/12/2005; accepted 5/13/2005.

Grant support: Grants DAMD 17-02-1-0012 (N.L. Weigel and W.E. Bingman), DAMD 17-01-1-0018 (I.U. Agoulnik), DK56252 (N.L. Weigel), and T32-DK07763 (I.U. Agoulnik), and NIH Specialized Program of Research Excellence in Prostate Cancer grant CA58204 (M.M. Ittmann, N.L. Weigel, G. Ayala, H. Erdeme, and A. Vaid).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

Role of SRC-1 in the Promotion of Prostate Cancer Cell Growth and Tumor Progression


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/17/7959

Cited articles
This article cites 33 articles, 18 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/17/7959.full#ref-list-1

Citing articles
This article has been cited by 29 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/17/7959.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.