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Targeted disruption of the p160 coactivator interface of androgen receptor (AR) selectively inhibits AR activity in both androgen-dependent and castration-resistant AR-expressing prostate cancer cells

Manjula Nakka^a, Irina U. Agoulnik^{a,b}, and Nancy L. Weigel^{a,*}

Manjula Nakka: Manjula.Nakka@bcm.edu; Irina U. Agoulnik: iagoulnik@fiu.edu; Nancy L. Weigel: nweigel@bcm.edu

^aDepartment of Molecular and Cellular Biology, Baylor College of Medicine

Abstract

The evidence that androgen blockade-resistant prostate cancer, termed castration resistant, remains androgen receptor (AR) dependent is compelling. AR is re-activated through multiple mechanisms including expression of constitutively active splice variants that lack hormone binding domains (HBD). This highlights the need to develop therapies that target regions other than the HBD. Because the p160 coactivators interact most strongly with the amino-terminus of AR, we examined the consequences of disrupting this interaction. We identified two overlapping SRC-1 peptides that interact with AR, but not with progesterone receptor. These peptides reduce AR and AR variant AR-V7 dependent induction of an AR responsive reporter. Using mammalian two hybrid assays, we found that the peptides interrupt the AR/SRC-1; AR/SRC-2 and AR N/C interactions, but not SRC-1/CARM-1 interactions. Consistent with the SRC-1 dependence of induced, but not repressed genes, in LNCaP cells, the peptides inhibited hormone dependent induction of endogenous target genes including PSA and TMPRSS2, but did not block AR dependent repression of UGT2B17 or inhibit vitamin D receptor activity. Simultaneous detection of SRC-1 peptides and PSA by double immunofluorescence in transfected LNCaP cells clearly demonstrated a strong reduction in PSA levels in cells expressing the peptides. The peptides also inhibited the AR dependent expression of PSA in castration resistant C4-2 cells,. Moreover they inhibited androgen dependent proliferation of LNCaP cells and proliferation of C4-2 cells in androgen depleted medium without affecting AR negative PC-3 cells. Thus, the p160 coactivator binding site is a novel potential therapeutic target to inhibit AR activity.

Keywords

SRC-1; androgen receptor; prostate cancer; peptide; CRPC

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*Corresponding Author at: Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, BCM130, Houston, TX 77030, USA. Tel.: +1 713 798 6234; fax: +1 713 790 1275.

^bDepartment of Cellular Biology and Pharmacology, Florida International University 11200 SW 8th Street, HLSI 419C, Miami, Florida 33199 (present address)

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1. Introduction

Prostate cancer (PCa), an androgen dependent disease, is the second most common cause of death from cancer in American men (American Cancer Society) (Jemal et al. 2010). Locally advanced and metastatic PCa are treated with some form of androgen blockade. Most tumors respond initially, but recur within two years. Androgens act through the androgen receptor (AR), a hormone activated transcription factor that binds to specific DNA sequences and recruits a series of coactivator complexes to modulate transcription of target genes (Mangelsdorf et al. 1995; Shang et al. 2002). Recurrent tumors, termed castration resistant PCa (CRPC) continue to rely on AR action despite reduced levels of circulating androgens (Agoulnik and Weigel, 2006). Recent studies show that some CRPC respond to abiraterone acetate, an inhibitor of adrenal and intratumoral synthesis of androgens, or to MDV3100, a novel non-steroidal anti-androgen, increasing overall survival by a few months in clinical trials (Potter et al. 1995; Tran et al. 2009).

Several mechanisms have been suggested for reactivation of AR. These include increased expression of AR, local synthesis of androgens, and changes in cell signaling or coactivator expression leading to AR activation (Agoulnik and Weigel, 2006). In addition, AR variants lacking the hormone binding domain are found in many CRPC and may contribute to resistance to current therapies (Dehm et al. 2008; Hu et al. 2009). This highlights the need for therapies that target other regions of AR or reduce overall expression. A previous study showed that over-expression of the region of AR that is amino terminal of the DNA binding domain was sufficient to inhibit androgen-dependent LNCaP xenograft growth (Quayle et al. 2007). This region contains the primary interaction site for steroid receptor coactivator-1 (SRC-1) and likely for the other related p160 coactivators, SRC-2 and SRC-3. In contrast to other steroid receptors, AR interacts weakly with LXXLL motifs of p160 coactivator proteins and interacts predominantly through a glutamine rich (Qr) region in the C-terminus of the p160 coactivators, which contain three small conserved regions termed A,B, and C (Bevan et al. 1999; Christiaens et al. 2002; Ma et al. 1999) (see Fig 1). Elevated levels of SRC-1 correlate with markers of more aggressive disease (Agoulnik et al. 2005) and tumors expressing high levels of SRC-2/NCoA2/TIF2 recur more quickly than those with low levels of SRC-2 expression (Agoulnik et al. 2006). Recently, Taylor et al. (Taylor et al. 2010), using genomic profiling, concluded that SRC-2/NCoA2 is an oncogene in 11% of PCa. Thus, we hypothesized that blocking the p160 interacting interface in AR should block AR activity regardless of receptor form or mode of activation. If this is correct, this surface could be a therapeutic target in CRPC. Previous studies have shown that SRC-1 lacking the LXXLL binding motifs retained the ability to interact with and coactivate AR (Bevan et al. 1999). Thus, we sought to determine whether blocking the amino-terminal coactivator binding site using a peptide derived from SRC-1 would be sufficient to block AR dependent transactivation and AR dependent cell growth without inhibiting the actions of related nuclear receptor family members.

2. Materials and methods

2.1. Reagents

R1881 (methyltrienolone) was purchased from PerkinElmer Life Sciences (Boston, MA), calcitriol (1,25(OH)₂D₃) from Solvay DuPhar (Weesp, The Netherlands). R5020 (promegestone) from NEN Life Science Products (Boston, MA) and [³H]thymidine from ICN (Irvine, CA). Serum, Triiodo thyronine (T3) and dexamethasone were purchased from Sigma (St. Louis, MO). AR was detected with mouse monoclonal AR 441 antibody (Nazareth et al. 1999) and PR with mouse monoclonal 1294 antibody (Press et al. 2002), kindly provided by Dr. Dean Edwards, Baylor College of Medicine (Houston, TX). The SRC-1 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), the

actin antibody from Chemicon (Temecula, CA), and the PSA antibody from DakoCytomation (Denmark). Bound antibodies were detected using ECL (enhanced chemiluminescence) kits from Amersham (Pharmacia Biotech, Inc. Piscataway, NJ). Tissue culture plastic ware was purchased from VWR (Sugarland, TX). All other chemicals were reagent grade.

2.2. Plasmids and plasmid construction

P100: To express P100 as a FLAG tagged peptide, the region corresponding to amino acids 1050–1150 of SRC-1 was PCR amplified using a primer containing a flag sequence and an EcoRI restriction site at the 5′ end and a primer containing a NotI restriction site at the 3′ end. Forward primer: CCCGAATTGCGCCACCATGGACTACAAGGACGACGATGACAAGCCTA ACCAGCTTCGACTTC. Reverse primer: GCCGCGGCCGCTATAGTCCAGATGAGGGAGG. After digestion with EcoRI and NotI, the PCR product was cloned into the EcoRI and NotI restrictions sites of pCR3.1 (Invitrogen, Carlsbad, CA). P200: The region of SRC-1 corresponding to amino acids 1050–1240 was PCR amplified and cloned into the pCR3.1 backbone as described for P100. The forward primer was the same as for P100. The reverse primer was GCCGCGGCCGCTATTGGGGAACCATTCCTGG. GalP100: To express the 1050–1145 region of SRC-1 as a GAL4 fusion peptide (GalP100), it was PCR amplified with primers containing Not I and Sal I restriction enzyme sites at the 5′ and 3′ ends and cloned into NotI and SalI of the pBIND vector (Promega, Madison, WI), which contains the yeast GAL4 DNA-binding domain upstream of a multiple cloning site. Forward primer: GGGGTCGACTTCCTAACCAGCTTCGACTTC. Reverse primer: GCCGCGGCCGCTAAGGGAGGTTGTTCCTAAA. GalP200: The region corresponding to amino acids 1050–1240 of SRC-1 was PCR amplified with Not I and Sal I restriction enzyme sites and cloned into the pBIND vector. The same forward primer as above was used. The reverse primer was GCCGCGGCCGCTATTGGGGAACCATTCCTGG. GSTP100: The region of SRC-1 corresponding to amino acids 1050–1150 was PCR amplified with primers containing EcoRI and NotI restriction sites at the 5′ and 3′ ends respectively and cloned into the EcoRI and NotI restriction sites of the pGEX-4T-1 vector (Amersham Biosciences). The forward and reverse primers were the same as for P100. GSTP200: The region of SRC-1 corresponding to amino acids 1050–1240 was PCR amplified and cloned into pGEX-4T-1 vector similar to GSTP100. The forward and reverse primers were the same as P200. pCR3.1. ARV7: First strand cDNA synthesis was done using 1000 ng total RNA from VCaP cells, 500 ng oligo(dT) and 200 units of SuperScript II reverse transcriptase (Invitrogen) in a volume of 20 μL. Biorad's iProof GC Master Mix was used to facilitate amplification of GC rich sequences along with 5% DMSO. The region corresponding to ARV7 (Guo et al. 2009) was PCR amplified using a primer containing a BamHI restriction site at the 5′ end and a primer containing an EcoRI restriction site at the 3′ end and cloned into the pCR3.1 vector backbone. Expression and reporter plasmids including pCR3.1 AR, pCR3.1 PR-B (Agoulunik et al. 2003), pCR3.1 SRC-1 (Jenster et al. 1997), 17mer luc, pBind SRC-1 (Rowan et al. 2000), GRE₂-E1b-LUC (Nawaz et al. 1999), VDRE-tk-LUC (a vitamin D receptor [VDR]-responsive promoter) (Zou et al. 1997), pCMV-βGal (Bai and Weigel, 1996) and pRShGRα (Expression vector for human glucocorticoid receptor) have been described earlier (Giguere et al. 1987). pAct-AR (Full length), pAct-ARNTD (AR amino terminus and DNA-binding domain, 1–660 aa) and GAL-ARDH (AR DNA and hormone-binding domains, 624–919 aa) were kindly provided by Dr. Elizabeth M. Wilson, University of North Carolina, (Langley et al. 1998). Plasmids pSG5-human TRβ2 and DR-4M-pTK-luciferase reporter were provided by Dr. Martin Privalsky, UC Davis (Vivanco-Ruiz et al. 1991). VP16 Coactivator associated arginine

methyltransferase-1 (CARM-1) was kindly provided by Dr. Michael Stallcup (University of Southern California).

2.3. Cell culture

HeLa and COS-1 cells were purchased from the American Type Culture Collection (ATCC) (Manassas, Va.) and were maintained in Dulbecco's modified Eagle medium (DME) plus 10% fetal bovine serum with penicillin-streptomycin (Invitrogen, Carlsbad, CA). AR-positive LNCaP prostate cancer cells from ATCC were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 containing 10% fetal bovine serum (FBS) with penicillin and streptomycin. The androgen independent C4-2 cell line was purchased from UroCor, Inc. (Oklahoma City, OK) and grown in T medium containing 5% FBS (Tsuchiya and Umeda, 1995). AR-negative PC-3 prostate cancer cells from ATCC were grown in DMEM/F12-10% FBS and penicillin/streptomycin. VCaP cells, a kind gift from Dr. Kenneth Pienta (University of Michigan), were maintained in DME plus 10% FBS with penicillin-streptomycin (Korenchuk et al. 2001). All cells were maintained at 37°C with 5% CO₂ in a tissue culture incubator.

2.4. Transient transfection assays

HeLa cells—Transfections were performed using a previously described method that uses inactivated polylysine coupled adenovirus as a carrier for the plasmids (Nazareth and Weigel, 1996). HeLa cells, plated at 180,000 cells per well in 6 well plates, were incubated with various combinations of plasmid DNA pre-incubated with polylysine coupled adenovirus and changed into DME medium plus 10% charcoal stripped serum. Vehicle or hormone was added 24 hrs post-transfection and cells harvested 24 hours later using Promega 1X lysis buffer. Luciferase assays were performed using a Luminoskan Ascent Luminometer (Thermo Lab Systems, Marietta, OH) and normalized to β -galactosidase measured using a 2-Nitrophenyl-D-galactopyranoside (Sigma) colorimetric assay and a Multi Skan Plus plate reader (Thermo Lab Systems, Marietta, OH).

LNCaP and PC-3 cells—Cells were plated at 60% confluency in 6 well plates, transfected 24 hours later with the specified DNA (3 μ g/well) plus lipofectin (Invitrogen, Carlsbad, CA) mixtures in serum free medium. After an overnight incubation cells were changed into medium supplemented with 10 % charcoal stripped FBS and treated with hormone or vehicle for an additional 24 hrs. RNA was extracted and qRT-PCR was done to measure mRNA levels. Alternatively, cells were split and re-plated for a cell proliferation assay.

C4-2 cells—Cells were plated at 30% confluency in polylysine coated 60 mm dishes. After an overnight incubation with lipofectin and the indicated DNA (4 μ g) mixtures in serum free medium as per manufacturer's protocol, the medium was changed to T-medium containing 5% charcoal-stripped FBS and treated with vehicle or R1881 for another 24 hrs. Cells were either harvested for RNA extraction or split 1:3 and re-plated for a cell proliferation assay.

2.5. GST assay

pCR3.1 AR and pCR3.1 PR-B plasmids were transfected into COS-1 cells using the lysine coupled adenovirus based method. The cells were treated with 10 nM R1881 (AR) or R5020 (PR-B) for 24 hrs and extracts prepared in Promega 1X lysis buffer. Purified glutathione S-transferase (GST) protein from Sigma (St. Louis, MO) or GST-P100 or GST-P200 crude extracts were incubated with a 1:1 slurry of Glutathione - Sepharose beads (Amersham Pharmacia Biotech [Piscataway, NJ]) in NETN buffer (20 mM Tris [pH 8.0], 50 mM NaCl, 1 mM EDTA [pH 8.0], 0.5% NP-40) at 4°C for 3 hrs. The beads were washed with NETN

buffer and incubated overnight with the COS cell lysate containing AR or PR-B at 4°C. The beads were washed with NETN buffer, extracted with 2× Laemmli buffer (Tris-glycine SDS sample loading buffer) and PR-B and AR detected by western blotting following SDS gel electrophoresis.

2.6. Mammalian two hybrid interaction assays

The mammalian two hybrid assay is based on the ability of a protein fused to the Gal4 transcription factor DNA binding domain (DBD) to recruit a protein linked to a VP16 activation domain to activate transcription of a reporter containing multimerized Gal4 DNA binding sites (17-mer-Luc). To test for interactions between AR and SRC-1 or its fragments, two hundred thousand HeLa cells were transfected with 250 ng of 17-mer Luc and the indicated combinations of 100 ng of pAct (VP16 activation domain control), pAct AR, pBind (Gal4DBD control), GalP100, GalP200 and pBind SRC-1 using the lysine coupled adenovirus method and transferred to medium containing charcoal stripped serum. Approximately 24 hrs post-transfection, vehicle (ethanol) or 10 nM R1881 was added. Cells were harvested 24 hrs later, and assayed for luciferase activity, which was normalized to protein concentration determined by Bradford assay (Bio-Rad, Hercules, CA).

2.7. Cell proliferation assay

LNCaP and PC-3 cells plated at 60% confluency in 6-well plates were transfected with 3 µg of pCR3.1, P100 or P200. After 24 hrs cells were split 1:3, re-plated, treated with vehicle or 10 nM R1881 overnight, and assayed for proliferation by [³H] thymidine incorporation (Cavarretta et al. 2002).

2.8. Western blot analysis

Protein levels were determined using a Bradford assay. AR (Nazareth et al. 1999), PR (Clemm et al. 2000) (20–30 ug/lane) or SRC-1 (Rowan et al. 2000) (50 ug) were resolved on SDS gels and detected by western blotting as described previously.

2.9. Real-time reverse transcriptase PCR analysis

RNA was extracted using TRIzol reagent (Invitrogen) and expression measured using real-time PCR analyses (ABI Prism 7500 sequence detector, Foster City, CA) and the previously described amplicons for PSA, PMEPA, SGK, TMPRSS2, SRC-1, and 18S (Agoulunik et al. 2005; Agoulunik et al. 2008; Boonyaratanakornkit et al. 2006). For UGT2B17, the primers and probe were GCCCCTGGATCGAGCAGT, ACCCGAAGGTGCTTGCT and 6FAM-TCTGGATTGAGTTTGTTCATGCGCCATAAA-TAMRA. CYP24A1 was detected using the following primers and probe: CCCAGCGGCTGGAGATC, CCGTAGCCTTCTTTGCGG and 6FAM-AACCGTGAAGGCCTATCGCGACT-TAMRA.

2.10. siRNA transfection

Fifty picomoles of either Ambion Smart pool siRNA specific for SRC-1 or Ambion noncoding control siRNA (Ambion, Austin, TX) were incubated with 10 µL Lipofectamine 2000 (Invitrogen) as per manufacturer's protocol and added to LNCaP cells at ~50% confluency in six-well plates in serum free medium. After overnight incubation, the medium was changed to RPMI supplemented with 10 % charcoal stripped FBS and treated with vehicle or 1 nM R1881 for another 24 hrs before RNA extractions and protein analyses were done.

2.11. Double immunofluorescence

LNCaP cells were plated at 30% confluency and grown on coverslips in six well plates and then transfected with 3 µg of pCR3.1, P100, or P200. Cells were treated with 10 nM R1881 the next morning, fixed in 4% formaldehyde 24 hrs later, and then incubated with anti-PSA (1: 500) and anti-FLAG (1: 2500 [Sigma]) antibodies simultaneously. Cells were then washed five times with blocking buffer (5% milk in TBST) and incubated with secondary antibodies Alexa Fluor 488 (*green*) goat anti-rabbit IgG conjugate and Alexa Fluor 594 (*red*) goat anti-mouse conjugate (Invitrogen). The coverslips were mounted in Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and fluorescence was detected using a Zeiss (Thornwood, NY) AxioPlan2 microscope. The optimal exposure was found for detection of the fluorescent signal in R1881 treated cells and the same exposure used for all samples.

2.12 Statistical analyses

One way ANOVA with Tukey's multiple comparison test was used to determine the results of luciferase assays with three or more groups. Two way ANOVA with Bonferroni multiple comparisons was used to determine the results of luciferase assays, qRT-PCR assays and cell proliferation assays with three or more groups in two vehicle versus hormone treated conditions. Statistical analyses were performed on raw data with GraphPad Prism 5.03 software (Graph-Pad Software, San Diego, USA). Significance was accepted at *P = 0.05.

3. RESULTS

3.1. Interaction of SRC-1 derived peptides with AR

The structure of SRC-1 and the regions cloned for these studies are shown in Fig 1A. Sequences corresponding to amino acids 1050–1150 and 1050–1240 were cloned into the pBIND vector to produce Gal4 DNA binding domain (DBD) fusion proteins and termed Gal P100 and Gal P200. The interaction of these fragments with AR was compared with the interaction of full length SRC-1 using a mammalian two hybrid assay. As predicted, both fragments as well as SRC-1, itself, interact with AR (Fig 1B), but only the full length SRC-1 interacts with the methyltransferase, CARM1, whose interaction site is reported to be carboxyl terminal to the AR interaction site (Chen et al. 1999). Note that the hormone dependence of the interaction with AR is a consequence of the requirement for hormone to translocate AR to the nucleus and is similar to previous studies (Bevan et al. 1999; Ma et al. 1999). Ma et al. (Ma et al. 1999) have used GST pull down assays to show that the amino-terminal region of AR interacts with in vitro translated SRC-1 fragments). To confirm the interaction between AR and our peptides as well as to test for receptor specificity, a GST pull down assay in the reverse direction was done. COS cell lysates containing either AR or progesterone receptor (PR) were incubated with glutathione beads bound to GST, GSTP100, or GSTP200, washed, eluted, and AR and PR detected by western blotting. The SRC-1 derived peptides interact with AR as expected, but not with the closely related PR (Fig 1C).

3.2. SRC-1 derived peptides inhibit interaction between AR and SRC-1/SRC-2 and between ARNTD and ARLBD

To determine whether the P100 and/or P200 peptides were sufficient to block interaction between SRC-1 and AR, we used a mammalian two hybrid assay and found that both inhibit the interaction (Fig 2A). Both P100 and P200 also reduce the interaction between SRC-2 and AR (Fig 2B). To determine whether full length SRC-1 inhibits interaction to a greater extent than P100 or P200, we compared inhibition in the presence of R1881. We found that both P100 and P200 can reduce AR interaction with SRC-1 to the same extent as full length SRC-1 suggesting that the LXXLL motifs cannot compensate for the occlusion of the Q rich region (Fig 2C). The p160 coactivators compete for interaction with AR blocking the

interaction of the AR hormone binding domain (HBD) with the AR NTD (He et al. 2002; Shen et al. 2005). P100 and P200 also inhibit this interaction (Fig. 2D); this suggests that it is the Q rich region of SRC-1 and its interaction with the amino-terminus of AR rather than binding of SRC-1 to the carboxyl terminus of AR that is important for inhibiting the AR interaction.

3.3. AR and AR variant (ARV7) mediated transcriptional activation is disrupted by SRC-1 derived peptides

To examine the contribution of the SRC-1 binding site to AR transcriptional activity, we measured the activity of full length AR or of its constitutively active splice variant AR-V7 in the presence of increasing amounts of plasmids encoding the peptides. Both P100 and P200 inhibit the hormone dependent activity of AR activity in transfected HeLa cells although P200 is more effective at lower levels of DNA (Fig 3A). The peptides also reduced ARV-7 activity (Fig 3B). In contrast, the activity of the glucocorticoid receptor (GR), whose interactions with SRC-1 are primarily through LXXLL motifs (Heery et al. 1997) was unaffected by P100. At high levels, P200 does reduce GR activity. This may be due to its larger size; it may inhibit some protein protein interactions important for GR activity (Fig 3C). Interestingly, thyroid hormone receptor isoform $\beta 2$ (TR $\beta 2$) has also been shown to interact with the glutamine rich region of SRC-1 (Privalsky et al. 2009). Neither P100 nor P200 inhibited TR $\beta 2$ activity (Fig 3D).

3.4. SRC-1 derived peptides attenuate endogenous AR but not VDR activity in LNCaP cells

To determine how effectively the peptides inhibit endogenous AR activity in LNCaP cells, cells were transfected with P100, P200, or empty vector and androgen dependent induction and repression of AR target genes compared. Both peptides significantly repressed all tested R1881 inducible genes (PSA, TMPRSS2, PMEPA1, SGK) (Fig 4A, B, C and D). We determined that the transfection efficiency was approximately 50% and the black bar denotes the expected remaining activity if the peptide were 100% effective in successfully transfected cells. In contrast, AR dependent repression of UGT2B17 was unaffected by these peptides (Fig 4E). SRC-1 also potentiates vitamin D receptor (VDR) activity by interacting with the HBD of VDR (Gill et al. 1998). Neither peptide reduced VDR mediated induction of CYP24A1 (Fig. 4F). C4-2 cells are CRPC cells derived from the LNCaP cell line under castrate conditions (Wu et al. 1994). We have shown previously that C4-2 cell growth and PSA expression are AR dependent in androgen depleted medium (Agoulnik et al. 2005). The basal level of PSA in C4-2 cells in androgen depleted medium is higher than in LNCaP under the same conditions. Both P100 and P200 inhibit PSA expression in C4-2 cells (Fig 4H). In contrast, SGK, whose basal expression is similar in LNCaP and C4-2 cells, predominantly shows a reduction in androgen dependent expression (Fig 4G) in this line.

3.5. Analysis of PSA inhibition at the single cell level

To assess relative efficacy of PSA inhibition in cells expressing the peptides, we used a double immunofluorescence technique. LNCaP cells transfected with vector (PCR3.1), P100, or P200 were stained for PSA (green) and flag (red) and nuclei detected with DAPI. Vector transfected cells stained strongly for PSA (Fig 5). In contrast, cells expressing high levels of P100 or P200 for the most part had greatly reduced levels of PSA (Fig. 5). To quantify the percent of cells expressing peptide that had substantially reduced PSA expression, we compared expression in cells expressing peptides with the total population of vector transfected cells. Examples 1 and 2 (Fig 5) show examples of cells that contain flag signal, but retain substantial amounts of PSA (designated as +) and examples of cells with reduced levels of PSA, which we designated as reduced PSA (-). The proportion of + and - cells in these fields is not representative since Flag +, PSA+ cells are relatively rare. Using these criteria, there were <5% low PSA containing cells in the vector transfected cells,

whereas about 80% of the P100 and P200 cells had low levels of PSA suggesting that the peptides were very effective in inhibiting AR action in the successfully transfected cells.

3.6. Endogenous SRC-1 is required for the expression of AR target genes sensitive to peptide inhibition

The peptide inhibitor results suggest that all of the induced genes require SRC-1 for optimal induction, but the repressed gene does not. SRC-1, itself, is a histone acetyltransferase and recruits other histone acetyltransferases such as p300/CBP (York and O'Malley, 2010). We have shown previously that SRC-1 is required for optimal PSA expression (Agoulunik et al. 2005) and in later papers that SRC-2 and SRC-3 are required for optimal induction of AR target genes (Agoulunik et al. 2006, Agoulunik and Weigel 2009) consistent with repression of this induction by the SRC-1 peptides. To determine whether the peptide resistant gene UGTB17 required SRC-1, LNCaP cells were treated with SRC-1 siRNA or control siRNA, treated with vehicle or R1881, and expression of AR target genes measured. SRC-1 mRNA levels were measured to confirm the effectiveness of SRC-1 siRNA (Fig 6A); there was more than a 60% decrease in SRC-1 mRNA and a substantial reduction in SRC-1 protein (Fig 6F). We had previously reported that optimal androgen dependent PSA induction required SRC-1 (Agoulunik et al. 2005) and similar results were obtained here (6B). SRC-1 also was required for optimal induction of SGK and PMEPA1 (6C and 6D). However, depletion of SRC-1 did not block the ability of AR to repress UGT2B17 expression. (Fig 6E).

3.6. SRC-1 derived peptides inhibit proliferation of AR dependent prostate cancer cell growth

Since optimal AR activity and SRC-1 expression are essential for proliferation of androgen dependent LNCaP prostate cancer cells, we hypothesized that expression of P100 and P200 in these prostate cancer cells should inhibit cell growth. To test this hypothesis, we transfected LNCaP cells with P100 and P200 and compared the rates of DNA synthesis. Both P100 and P200 impede LNCaP cell growth (Fig 7A). If the decrease in cellular proliferation is a result of a reduction in AR activity, then these peptides should have no effect on the growth of PC-3 cells which lack AR. The result shown in Fig 7B confirms that the peptides do not reduce PC-3 cell proliferation. Further, the [³H] thymidine incorporation assay results indicate that P100 and P200 inhibit the proliferation of C4-2 in hormone depleted medium (Fig 7C).

4. DISCUSSION

The mounting evidence that CRPC tumors remain AR dependent coupled with the finding that constitutively active splice variants of AR lacking the HBD are expressed in CRPC underscores the need for agents that target other domains of AR. The reports that AR primarily interacts with a glutamine rich region in p160 coactivators rather than the LXXLL motifs suggested that occluding the AR interaction site would inhibit AR activity regardless of isoform or mode of activation. Our finding that the P100 and P200 peptides can compete for AR/SRC-1 interaction to the same extent as full length SRC-1 suggests that interactions with the LXXLL motifs are weak and cannot compensate for the loss of the primary interaction. Moreover, the peptides inhibit interaction between AR and SRC-2/NCoA2, a coactivator that is amplified and/or overexpressed in 11% of prostate cancers (Taylor et al. 2010). This is not surprising because the three p160 coactivators exhibit substantial homology in this region (Christiaens et al. 2002), and the relatively large size of the peptide would block interactions with proteins with overlapping binding sites. Steroid receptors recruit a series of coactivators to remodel chromatin and regulate transcription. Our studies indicate that the p160 coactivators are not required for repression of at least some AR target

genes, but that repression of these targets also is not required to inhibit AR dependent growth. P100 and P200 inhibit the activity of AR in the castration resistant C4-2 cells as well as the activity of the constitutively active variant, AR-V7. Thus, this interaction domain provides a new therapeutic target for castration resistant prostate cancer.

Although the hormone binding domain has been the primary target for the development of AR inhibitors, there have been some studies seeking to limit AR action through targeting other domains. EPI-001, a metabolite of bisphenol A, inhibits AR activity and is particularly effective in inhibiting activity induced by forskolin or IL-6 (Andersen et al. 2010). This compound reduces DNA binding and limits binding to CBP. Another small molecule, harmol, inhibits AR activity, does not compete with agonist binding to the ligand binding domain, but does reduce AR binding to chromatin (Jones et al. 2009). The mechanism of action of P100 and P200 appears to differ in that AR continues to repress target gene expression indicating that AR retains its capacity to bind to DNA. These peptides exhibit specificity for interaction with AR and inhibition of AR mediated transcription without inhibition of the activities of several other nuclear receptors. Taken together with the lack of effect on growth of AR negative PC-3 cells, either the peptides themselves or a small molecule mimic are candidates to block AR action in CRPC with minimal side effects.

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Abbreviations

ANOVA	analysis of variance
AR	Androgen Receptor
ARDH	AR DNA and hormone-binding domains
ARNTD	AR amino terminus and DNA-binding domain
ATCC	American Type Culture Collection
CARM-1	Coactivator Associated arginine methyltransferase-1
CRPC	Castration resistance prostate cancer
DME	Dulbecco's modified Eagle medium
FBS	Fetal bovine serum
ECL	enhanced chemiluminescence
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GST	glutathione S-transferase
HBD	hormone binding domain
luc	Luciferase
PCa	Prostate Cancer

PR	Progesterone Receptor
Qr	glutamine rich
RLU	relative light units
RPMI	Roswell Park Memorial Institute medium
RT-PCR	reverse transcription-PCR
SGK1	serum and glucocorticoid regulated kinase-1
siRNA	small interfering RNA
SRC-1	Steroid Receptor Coactivator
TRβ2	thyroid hormone receptor isoform β 2
VDR	Vitamin D Receptor

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FIGURE 1. Design and initial tests of peptides

A, Structure of SRC-1 including the locations of the LXXLL motifs and the location of the Q rich region (989–1240 amino acids) consisting of the A box (1050–1099), the B box (1101–1135) and the C box (1160–1177) box. Also shown are the regions corresponding to the P100 (1050–1150) and P200 (1050–1240) peptides and the corresponding Gal fusion proteins. B, Interaction of AR and SRC-1 fragments in a mammalian two hybrid assay. HeLa cells were transfected with 250 ng of the Gal4 responsive 17-mer Luc and the indicated combinations of either 100 ng of Act (VP16 control), Act AR, Bind (Gal4DBD control), Gal P100, Gal P200, Bind SRC-1 and Act CARM-1, treated with vehicle or 10 nM R1881, harvested 24 hrs later, luciferase activity measured and normalized to protein. Each condition was tested in triplicate and average and SEM were calculated. The experiment was done more than three times and a representative experiment is shown. C, 1 μ g of pCR3.1 AR or pCR3.1 PR-B was transfected into COS cells, treated with 10 nM R1881 or 10 nM R5020, cells harvested, and lysed with Promega 1X lysis buffer containing protease inhibitors. Equal amounts of extract were incubated with beads containing GST, GST P100, or GST P200, washed, bound protein eluted and AR and PR detected by western blotting. The experiment was done three times and a representative experiment is shown. ***p<0.001 one way ANOVA with Tukey's multiple comparison test, bind/aAR vs. aAR/bSRC-1, bind/aAR vs. aAR/Gal P100, bind/aAR vs. aAR/GalP200 (in the presence of R1881).

FIGURE 2. The peptides inhibit AR- SRC-1/SRC-2 interactions and AR N/C interactions

A, B, C, D, Mammalian two hybrid assays were performed using 250 ng of 17-mer Luc and the indicated combinations of either 100 ng of Act, Act AR, Act ARNTD, Bind, Bind ARDH, Bind SRC-2 and Bind SRC-1 with increasing amounts of P100, P200, or SRC-1 (100 ng, 500 ng, 800 ng) transfected into HeLa cells. The pCR3.1 vector was used to equalize DNA amounts transfected. Vehicle or 10 nM R1881 was added 24 hours post-transfection and cells were harvested for luciferase assays 24 hours later. Each condition was tested in triplicate and average and SEM values were calculated. Each experiment was done at least three times and a representative experiment is shown. A, inhibition of the interaction between AR and SRC-1. * $p < 0.05$; *** $p < 0.001$ one way ANOVA with Tukey's multiple comparison test, aAR/bSRC-1 vs. aAR/bSRC-1 plus P100 or P200 in the presence of R1881. B, inhibition of interaction between AR and SRC-2. *** $p < 0.001$ one way ANOVA with Tukey's multiple comparison test, aAR/bSRC-2 vs. aAR/bSRC-2 plus P100

or P200 in the presence of R1881. C, comparison of efficacy of inhibition by SRC-1, P100, and P200. * $p < 0.05$; *** $p < 0.001$ one way ANOVA with Tukey's multiple comparison test, aAR/bSRC-1 vs. aAR/bSRC-1 plus SRC-1, P100 or P200 in presence of R1881. D, Inhibition of AR N/C interactions. *** $p < 0.001$ one way ANOVA with Tukey's multiple comparison test, aARNTD/bARDH vs. aARNTD/bARDH plus P100 or P200 in the presence of R1881.

FIGURE 3. SRC-1 peptide inhibitors selectively inhibit AR and AR variant (AR-V7) activity
 A, B, HeLa cells were transfected with either 0.3 ng androgen receptor (pCR3.1 AR) or 0.5 ng AR-V7, 250 ng of GRE2E1b luc reporter, P100/P200 (ranging from 100 ng to 1250 ng) and 50 ng of pCMV-βGal vectors. Cells were treated or not with 10 nM R1881 for 24 hrs and relative luciferase activities were measured and normalized to β galactosidase activity. Each data point was done in triplicate and average and SEM values were calculated. For fig 3A, ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ two way ANOVA with Bonferroni multiple comparisons, AR vs. AR plus P100 or P200 in the presence of R1881. For fig 3B, *** $p < 0.001$ one way ANOVA with Tukey's multiple comparison test, ARV7 vs. ARV7 plus P100 or P200. C and D, Similar to A and B, hormone dependent luciferase activity was measured in these experiments to investigate GR and TRβ2 activity. HeLa cells were transfected with either 1ng of glucocorticoid receptor (pRShGRα) with 250 ng of GRE2E1b luc reporter or 20 ng of TRβ2 with 250 ng of DR-4-tk-luciferase reporter, P100 or P200 (ranging from 100 ng to 1000 ng) and 50 ng of pCMV-βGal vectors. 24 hrs post-transfection, cells were treated or not with 100 nM dexamethasone (for GR) or 100 nM T3 (for TRβ2) for 24 hrs and relative luciferase activities were measured both in the presence and absence of hormone. RLU values were normalized to β galactosidase activity. Each experiment was done more than three times and a representative experiment is shown. For fig 3C, *** $p < 0.001$; **** $p < 0.0001$ two way ANOVA with Bonferroni multiple comparisons, GR vs. GR plus P100 or P200 in the presence of Dexamethasone.

FIGURE 4. P100 and P200 inhibit AR target gene expression

A–E, LNCaP cells were transfected with 3 μ g of pCR3.1, P100 or P200, with vehicle or 10 nM R1881 24 hrs post transfection and harvested 24 hrs later. RNA was extracted and analyzed by qRT-PCR. Each condition was done in triplicate and average and SEM values were calculated. Each experiment was done at least three times and a representative experiment is shown. The values for cells transfected with pCR3.1 and treated with R1881 were set at 100 and other values reported relative to this value. ****p<0.0001; ***p<0.001; **p<0.01; two way ANOVA with Bonferroni multiple comparisons, PCR3.1 vs. P100 or P200 in presence of R1881. F, LNCaP cells were transfected with 3 μ g of pCR3.1, P100 or P200 as in A–E. Cells were treated with vehicle or 100 nM 1,25D 24 hrs post transfection and harvested 24 hrs later. RNA was extracted and CYP24A1 measured by quantitative RTPCR. G and H, C4-2 cells were plated in polylysine coated plates and transfected with 3 μ g of pCR3.1, P100 or P200. Twenty four hours post-transfection cells were treated with 10 nM R1881 for another 24 hrs, harvested, RNA was extracted and analyzed by qRT-PCR. Each data point was done in triplicate and average and SEM values were calculated. Each experiment was done more than three times and a representative experiment is shown. *p<0.05; **p<0.01; ****p<0.0001; two way ANOVA with Bonferroni multiple comparisons, PCR3.1 vs. P100 or P200 in presence of R1881.

Figure 5. P100 and P200 reduce PSA protein expression

A, LNCaP cells were plated on coverslips in 6 well plates and transfected with 3 μ g of pCR3.1, P100, or P200. Cells were then treated with 10 nM R1881 for 24 hrs and fixed. Cells were incubated with anti-PSA and anti-Flag antibodies simultaneously overnight at 4°C followed by incubation with FITC labeled secondary antibody (PSA detection) and Texas red labeled secondary antibody (P100 and P200 detection). Fluorescent images were taken with a Zeiss axioplan microscope and images were captured in Adobe Photoshop 4.0. B, Approximately 150 cells were counted for each plasmid population and the percentage of cells with normal or greatly reduced PSA expression were plotted. For P100 and P200, only cells staining positive for flag signal were counted. SD was determined from the average and variation between different fields for each population. Examples 1 and 2 show examples of flag expressing cells judged to have greatly reduced PSA (–) and those that have significant amounts of residual PSA (+). Columns labeled P100, P200 and PCR3.1 were transfected with the corresponding plasmids. Both P100 and P200 expressing cells show minimal residual PSA. Scale bar is 50 μ m, ****p<0.0001. ****p<0.0001 two way ANOVA with Bonferroni multiple comparisons, PSA with no peptide vs. PSA plus P100 or P200.

FIGURE 6. Depletion of SRC-1 reduces expression of genes induced by R1881, but does not prevent R1881 mediated repression

LNCaP cells were plated at 50% confluency and then transfected with non-coding or SRC-1 specific siRNAs. Cells were treated with 1 nM R1881 and harvested 24 hrs later. RNA was extracted and qRT-PCR was performed and mRNA levels of AR target genes were measured, normalized to 18S levels and R1881 treated control siRNA cell values set at 100 (A-E). F, Western blot was performed on samples transfected in parallel and SRC-1 and actin levels were determined. For A-E, each data point was done in triplicate and average and SEM values were calculated. Each experiment was done more than three times and a representative experiment is shown. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ two way ANOVA with Bonferroni multiple comparisons, CONsi vs. SRC-1si.

FIGURE 7. P100 and P200 selectively inhibit proliferation of AR positive prostate cancer cells LNCaP (A) and PC-3 cells (B) were transfected with 3 μg of pCR3.1, P100 or P200. After 48 hrs cells were split 1:3 and replated, LNCaP cells were treated with vehicle or R1881 overnight and both LNCaP and PC-3 cells assayed for $[^3\text{H}]$ thymidine incorporation. Each data point was done in triplicate and average and SEM values were calculated. Each experiment was done more than three times and a representative experiment is shown. C, C4-2 cells were plated in polylysine coated plates at 20% confluency and then transfected with 3 μg of pCR3.1, P100 or P200. After 24 hrs cells were treated with vehicle or hormone for another 48 hrs. Cells were then assayed for $[^3\text{H}]$ thymidine incorporation. *** $p < 0.001$; **** $p < 0.0001$ two way ANOVA with Bonferroni multiple comparisons, PCR3.1 vs. P100 or P200.