ANDROGEN RECEPTOR AND EPIDERMAL GROWTH FACTOR SIGNALING IN PROSTATE CANCER

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ABSTRACT

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Epidermal Growth Factor-Meditated Signaling in Recurrent Prostate Cancer

(Under the direction of Elizabeth M. Wilson, Ph.D)

The androgen receptor (AR) is required for prostate cancer development and contributes to tumor progression following remission in response to androgen deprivation therapy. Epidermal growth factor (EGF) increases AR transcriptional activity at low levels of androgen in the CWR-R1 prostate cancer cell line derived from the castration-recurrent CWR22 prostate cancer xenograft. Our studies indicated that EGF does not regulate TIF2 mRNA levels and that TIF2 is required for AR transactivation induced by DHT and EGF in CWR-R1 cells. Real-time RT-PCR was used to determine that hK2 is a suitable marker for androgen-regulated gene transcription in CWR-R1 cells and that DHT does not up-regulate EGF transcription. Here we report that knockdown of AR decreases EGF stimulation of prostate cancer cell growth and demonstrate a mechanistic link between EGF and AR signaling. The EGF induced increase in AR transcriptional activity is dependent on phosphorylation at MAP kinase consensus site Ser-515 in the AR NH_2-terminal region and at protein kinase C consensus site Ser-578 in the AR DNA binding domain. Phosphorylation at these sites alters the nuclear-cytoplasmic shuttling of AR and AR interaction with the Ku-70/80 regulatory subunits of DNA-dependent protein kinase. Abolishing AR Ser-578 phosphorylation by introducing an S578A mutation eliminates the AR transcriptional response to EGF, increases both AR binding of Ku-70/80 and

nuclear retention of AR in association with hyperphosphorylation of AR Ser-515. AR-S578A did not transactivate the MMTV-Luc reporter and we show the negative regulatory element-1 in the long terminal repeat of MMTV mediates AR-induced transcription of that promoter. The results support a model in which AR transcriptional activity increases castration-recurrent prostate cancer cell growth in response to EGF by site-specific serine phosphorylation that regulates nuclear-cytoplasmic shuttling through interactions with the Ku-70/80 regulatory complex.

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LIST OF ABBREVIATIONS

AIB1- amplified in breast cancer-1

AKT-protein kinase B

AR- androgen receptor

BPH- benign prostate hyperplasia

CARM-1- Coactivator Associated Arginine Methyl Transferase

CMV- cytomegalovirus

COS- monkey kindney cells

CWR22- Case Western Reserve

DHT- dihydrotestosterone

DNA-PK- DNA-dependent protein kinase

EGF- epidermal growth factor

EGFR- epidermal growth factor receptor

GFP- green fluorescent protein

GusB- glucuronidase B

HER- human epidermal growth factor receptor

HEK293 - human embryonic kidney cells

hK2- human kallikrein 2

HRG- heregulin

IGF-1- insulin growth factor 1

IGFBP5- insulin-like growth factor binding protein-5

IL-6- Interleukin 6

KGF- keratinocyte growth factor

MAGE-11- Melanoma antigen gene protein 11

MAPK- mitogen associated protein kinase

MMTV- mammary mouse tumor virus

N/C interaction- NH_2-terminal/carboxy terminal interaction

PIN- Prostatic intraepithelial neoplasia

PI3K- phosphatidylinositol-3-kinase

PKC- protein kinase C

PSA-prostate specific antigen

RFP- red fluorescent protein

RTK- receptor tyrosine kinase

scAAV- self-complementary adeno-associated viral vector

siRNA- silencing RNA

SRC- steroid receptor coactivator 1

SRC-3- steroid receptor coactivator 3

T- testosterone

TIF2- transcription intermediary factor 2

TNF-α- tumor necrosis factor alpha

VEGF- vascular endothelial growth factor

INTRODUCTION

PROSTATE CANCER: EPIDEMIOLOGY, NATURAL HISTORY AND CURRENT TREATMENTS

The prostate gland is the most common site of neoplastic disorders in men with 50% developing benign prostate hyperplasia (BPH) by the age of 50. At 80 years of age BPH is present in approximately 90% of men and 23% will develop prostate cancer (1). According to the American Cancer Society in 2008 about 186,320 new cases of prostate cancer will be diagnosed in the United States and 28,660 men will die from it, accounting for about 9% of cancer-related deaths. The incidence of prostate cancer extends also to other Western countries where it is the most prevalent male malignancy and ranks among the top three causes of death due to cancer (2). The pathogenesis of BPH and prostate cancer is poorly understood (3).

Epidemiologic studies on prostate cancer risk factors point to endocrine function, growth factors, diet, occupational exposures and genetic factors as possible etiologies of the disease (4). Recent cohort studies have identified multiple loci with moderate effects associated with susceptibility to prostate cancer. One of the largest, most recent genome-wide association studies identified copine III (CPNE3) of the calcium-dependent phospholipid-binding proteins, interleukin-16 (IL16) and cadherin 13 (CDH13) as possible prostate cancer susceptibility genes (5). Polymorphisms of breast cancer genes

1/2 (BRAC 1/2) and CHK2 checkpoint homolog (CHEK2), involved in DNA repair, have also been recently associated with prostate cancer development (6,7). Supporting evidence is required to determine the role of identified genetic polymorphisms and their potential interaction with environmental factors in the pathogenesis of prostate cancer.

Despite the number of candidate susceptibility genes involving multiple cellular pathways, the natural history of prostate cancer has been consistently linked to androgen signaling and the androgen receptor (AR), a member of the steroid superfamily of transcription factors. Androgens support the development and survival of normal and malignant prostate tissue by binding and transcriptionally activating AR. Polymorphisms of several genes directly involved in androgen synthesis, such as CYP11A1, CYP17A1/A2, and CYP19A1 from the cytochrome P450 family, are under study as biomarkers for prostate cancer susceptibility (8-11). Further, reports point to polymorphisms in AR (12-15) and AR coactivators, such as amplified in breast cancer-1 (AIB1) and steroid receptor coactivator 3 (SRC-3) (16), as increased risk factors of prostate cancer development.

The normal prostate is comprised of mature secretory luminal, prostatic basal, and neuroendocrine cells. The luminal or glandular cells constitute the exocrine compartment of the prostate and are terminally differentiated. Glandular cells express high levels of AR and are dependent on androgen for survival (17). Basal cells rest on the basement membrane of the prostate, are relatively undifferentiated and lack secretory activity. Unlike glandular cells, stromal cells express low levels of AR and stromal-epithelial cell interactions that modulate AR-coregulator recruitment and AR function seem altered in the stromal cell microenvironment of prostate cancer (18). Thus, although the great majority of cancers originate from gland cell disruption of androgen-regulated pathways,

both stromal and epithelial cell compartments may affect the development and progression of prostate cancer.

Neuroendocrine cells reside among the secretory epithelium in the normal prostate gland, are terminally differentiated and androgen-insensitive (19). Expression of VEGF leading to angiogenesis has been linked to oncogenic activity of neuroendocrine cells in the prostate (20). Transformed epithelial cells actively influence stromal cells, including inflammatory cells, vascular endothelial cells, and fibroblasts, to generate a microenvironment that fosters carcinogenesis (18) resulting in complex paracrine regulatory mechanisms during cancer development.

During the process of malignant transformation, cells gradually progress towards the malignant phenotype. Prostatic intraepithelial neoplasia (PIN), defined as a condition of neoplastic growth of epithelial cells with preexisting benign acini or ducts, is widely accepted as a precursor to prostate cancer (21). Studies suggest that high grade PIN represents an intermediate stage between pathological and phenotypical benign epithelium and the invasive malignant carcinoma (22-24). High grade PIN diagnosis is of clinical significance as it identifies patients at risk of malignancy and the development of concurrent carcinoma.

Initial evaluation of the extent of patient longevity involves clinical variables that correlate with the extent of disease. The probability of cure depends on the Gleason score, prostate-specific antigen (PSA) level and clinical stage (25). PSA is a serine protease believed to liquefy the seminal fluid (26). Prostate cancer is associated with increased release of PSA into the circulation, increasing the level in blood by up to 100,000-fold (27). PSA testing has come into widespread use as a prostate cancer marker, both for initial diagnosis and monitoring response to treatment. Use of PSA has also aided in the

prediction of prostate cancer risk and treatment outcome (28). Another prostate cancer marker under investigation is human kallikrein 2 (hK2) that, like PSA, is androgen regulated, released into seminal fluid, and increased in serum of patients with prostate cancer. Several studies have shown that measuring hK2 aids in predicting prostate cancer stage, grade and volume and progression to castration-recurrent cancer (29,30). Current research is aimed at establishing and developing methods to improve on the conventional PSA test to significantly reduce prostate cancer mortality.

Surgical management of localized or non-metastatic prostate cancer includes radical prostatectomy and cryotherapy (controlled freezing) of the prostate in order to destroy cancerous cells. Radiotherapy or small pellet radioactive implants (brachytherapy) is also used in patients with early stage disease. A number of novel agents are being explored to manage patients with advanced prostate cancer, both alone and especially in combination. Anti-angiogenic agents, small molecule inhibitors, and nucleotide-based targeted therapies are aimed at reducing prostate cancer mortality. Markers for tumor angiogenesis, such as microvessel density, have been reported to be greater in tumors from men with metastatic prostate cancer than in those with localized disease (31). Humanized monoclonal antibody that neutralizes vascular endothelial growth factor (VEGF) activity in combination with docetaxel, prednisone, and thalidomide has resulted in a high durable 86% response in lowering PSA levels. Small molecule inhibitors that specifically block cell signaling involved in growth and apoptosis are currently under investigation. For instance, Endothelin-A (ET-A) receptor activation by endothelin-1 (ET-1) contributes to tumor growth and progression in prostate cancer (32). ETA-1 receptor antagonist delays median time to PSA progression and prolongs median time of progression in castration-recurrent prostate cancer patients (33).

Antisense oligonucleotides are currently being explored to target genes involved in cancer progression (34). Antisense oligonucleotides toward protein kinase C (PKC), insulin growth factor binding protein 2, protein kinase A and the B-cell leukemia/lymphoma 2 genes are some of the targeting agents that have been studiend in clinical trials (35). Although antisense oligonucleotide chemistry holds potential clinical advantages, challenges remain to optimize tissue exposure and cellular uptake and to demonstrate the underlying mechanism and anti-tumor activity.

Since androgen activation of AR regulates prostate growth, drug treatment modalities are also largely targeted to blocking AR-mediated gene transcription. Circulating testosterone levels can be reduced by removing the testes (bilateral orchiectomy) or by chemical castration. A second treatment option is the interference with adrenal hormones in addition to testicular testosterone (combined androgen blockade). Greater understanding of the etiology, pathogenesis and molecular mechanisms of prostate cancer development and AR function are required for the development of more effective therapies to reduce prostate cancer mortality.

THE ANDROGEN RECEPTOR AND ANDROGEN RECEPTOR COACTIVATORS

Androgen action in target cells is mediated by the AR which exhibits a prototypic multidomain structure, containing a N-terminal activation domain, a C-terminal ligand-binding domain, and a centrally located DNA-binding region with two zinc finger motifs (36,37). The AR gene is located on the X chromosome at Xq11-12 (38). Androgen-induced AR transcriptional activity depends on activation function 1 in the disordered NH_2-terminal region (37) and activation function 2 (AF2), a highly ordered hydrophobic

surface in the ligand binding domain (LBD) that requires androgen binding for its structural integrity (39). AR AF2 binds a number of L*XX*LL-related motifs such as the AR NH_2-terminal F*XX*LF motif ^{23}FQNLF^{27} in an androgen-dependent and specific manner (40-42). AR F*XX*LF motif binding to AF2 is the basis for the AR NH_2- and carboxyl-terminal (N/C) interaction that contributes to AR dimerization (43,44) and is critical for AR regulation of androgen-dependent genes (45) (Figure 1.1).

AF2 in the ligand binding domain functions as a binding site for SRC/p160 coactivator LXXLL motifs and for the FXXLF motif present in the AR-NH_2 terminus and AR coregulators (40,46,47). Cell- and tissue-specific coactivators influence the contribution of AF2 to AR transcriptional activity. For instance, AR AF2 activity in prostate cancer has been linked to higher levels of SRC/p160 coactivators that compete for the AR N/C interaction and increase AR transcriptional activity through AF2 (48,49). Interestingly, transcriptional intermediary factor 2 (TIF2) is highly expressed in the majority of advanced castration-recurrent prostate cancers (49). Coactivators can enhance transcription of AR dependent genes. Gregory et al. reported that in the CWR22 xenograft, AR associated protein ARA70 is regulated by androgens and that its levels increase during prostate cancer recurrence (50). Bai et al. have recently shown that the

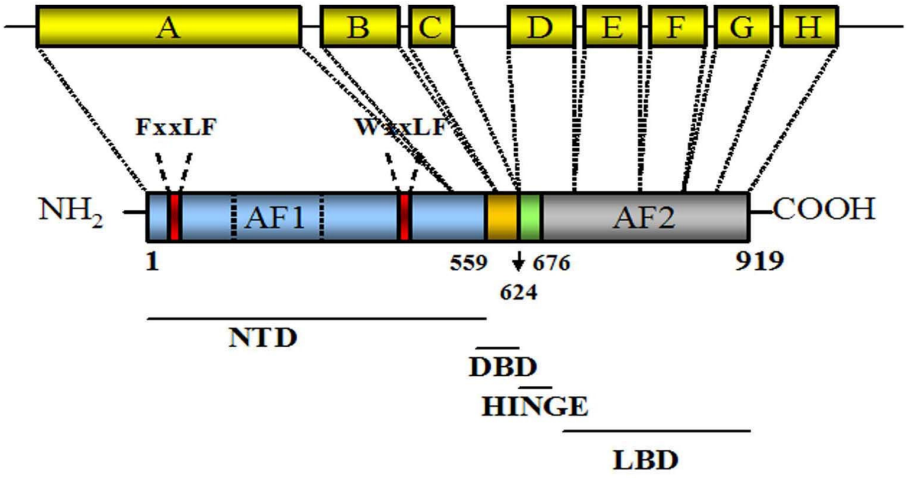

Figure 1.1. Modular structure of AR and functional domains. Eight exons code for a N-terminal activation domain, a C-terminal ligand-binding domain, hinge region and a centrally located DNA-binding region with two zinc finger motifs. Activation function 1 and 2 (AF1/2) are involved in the androgen-induced transcriptional activity of AR. The agonist-induced androgen receptor NH_2- and COOH-terminal (N/C) interaction is mediated by the F*XX*LF and W*XX*LF NH_2-terminal motifs.

AR coregulator melanoma antigen gene protein-11 (MAGE-11) of the MAGEA gene family binds the AR F*XX*LF motif to expose AF2 and increase coactivator recruitment. MAGE-11 is expressed in androgen-dependent tissues and in prostate cancer cell lines (46). Thus, coactivators have an important role in AR action contributing to prostate tumor development and progression. Overexpression of coactivators facilitates AR transactivation particularly at low levels of circulating androgen (51,52).

In reporter gene assays, androgen binding results in AR nuclear translocation and up-regulation of androgen responsive reporter genes through its interaction with androgen response elements (53). The major male sex steroids, testosterone (T) and dihydrotestosterone (DHT), initiate prostate gland growth and development. Testosterone is produced mainly by testicular Leydig cells and is converted by type II 5α-reductase in the prostate to DHT. Normal levels of T without conversion to DHT fail to stimulate complete male genital development of the human fetus as evidenced by the 5α-reductase syndrome caused by a genetic defect in the enzyme that converts T to DHT (54). Although T and DHT equilibrium binding affinities are similar (55), a ~10-fold higher concentration of T is required to achieve the AR mediated transcriptional effects of DHT (56). Ligand-specific effects of T and DHT result from different physiological potencies, where the weaker activity of T, a more polarized steroid than DHT, results in an inability to fully stabilize the AF2 binding surface for AR F*XX*LF and SRC/p160 coactivator L*XX*LL motif binding (52). Inhibition of 5α-reductase isoenzymes to decrease DHT has demonstrated benefits in the primary prevention of prostate cancer and potential in limiting disease progression in men with diagnosed disease (57). AR and coregulators bind to specific androgen response elements in the upstream promoter of target genes leading to transcriptional activation and modulation of RNA polymerase II (18,58).

Androgen regulation gene expression is controlled by a complex transcriptional machinery involving multiple regulatory signals and proteins. Understanding the underlying molecular mechanisms of AR function is critical to the development of effective prostate cancer treatments.

CASTRATION-RECURRENT PROSTATE CANCER

AR plays a key role in the development and maintenance of normal prostate and in prostate cancer progression. Alterations in AR itself, as well as interactions with growth factors, cofactors, chromatin, post-transcriptional modification mechanisms and degradation patterns, can alter AR function providing a selective growth advantage for cancer cells (59). Studies suggest amplifications of the AR gene make prostate cancer tumors hypersensitive to low levels of androgen. Studies have demonstrated mutations in AR result in promiscuity of the response to ligands and antagonists. Other groups have reported alterations in kinase signaling lead to an outlaw AR that is responsive to growth factor- and kinase-mediated signaling. Studies also indicate paracrine signals from the prostatic stroma result in AR transactivation in the presence of low levels of androgen (60) (Figure 1.2). The majority of men with non-organ confined prostate cancer initially experience regression in response to therapy but virtually all patients relapse and no longer respond to conventional anti-neoplastic therapy (61).

AR is expressed in the majority of castration-recurrent prostate cancers (62) and activation of AR contributes to malignant growth despite androgen deprivation. Visakorpi et al. reported amplification of the AR gene in 30% of the castration-recurrent tumors compared to the specimens taken from the same patients prior to androgen

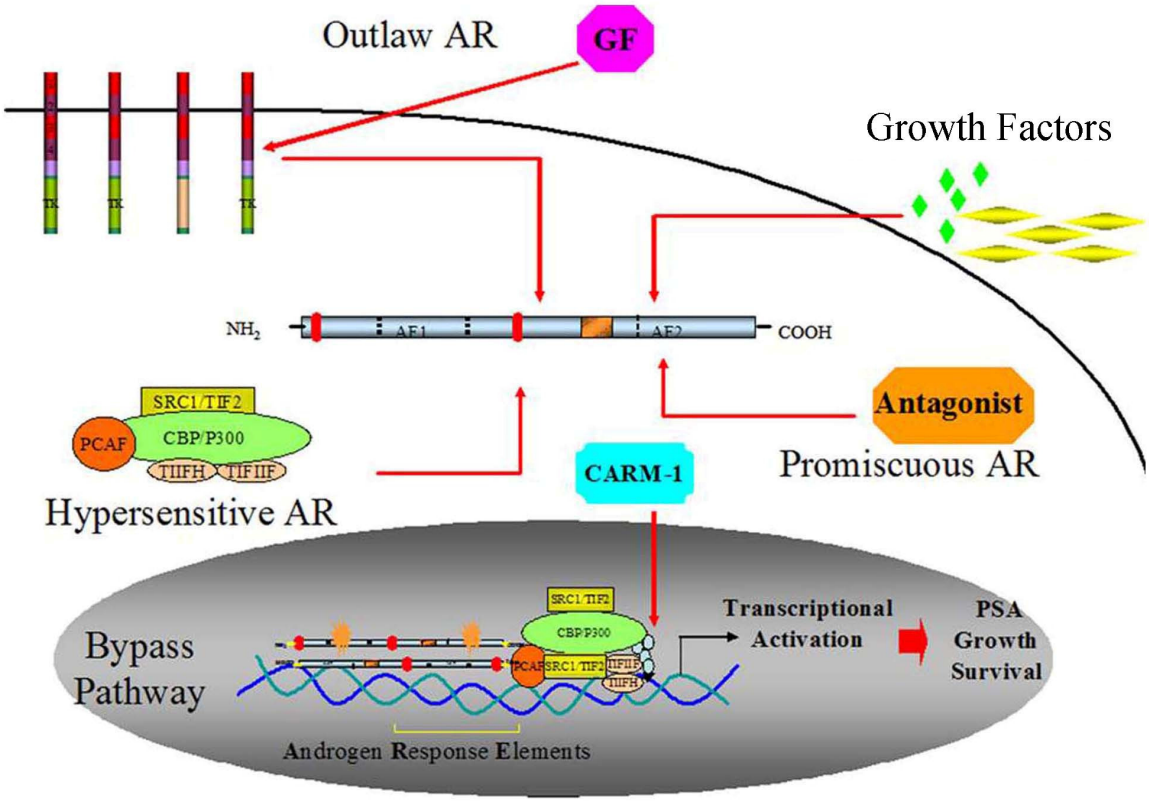

Figure 1.2. Molecular mechanism of castration-recurrent prostate cancer. Mechanisms associated with continued signaling through the androgen receptor despite castration include 1) changes in the levels of ligand(s) in tumor tissue, (2) increased levels of the protein due to gene amplification due to gene amplifications or alterations in mRNA regulation, (3) mutations in AR that lead to changes in structure and function, (4) increase expression or activity of coregulatory or coactivator proteins, (5) increases in AR activity after androgen deprivation by methyltransferases like CARM-1, and (6) factors that lead to activation of AR in the absence of ligand allowing kinase croos-talk (adapted from reference 60).

ablation therapy (63). These studies have been verified at the genomic, RNA and protein levels in castration-recurrent tumors (63-65).

Heightened sensitivity of AR to androgens has been proposed as another mechanism by which prostate cancer cells proliferate in an androgen-reduced environment. Studies by our group showed that the concentration of DHT required for growth stimulation of hormone refractory prostate cancer is four orders of magnitude lower than that required by androgen dependent cells and that, in the castration-recurrent prostate cancer cell line, CWR-R1 (derived from the CWR22 human prostate cancer xenograft) the AR is highly expressed, stable and localized to the nucleus (66). The human prostate cancer xenograft CWR22 propagated in nude mice maintains characteristics of human prostate cancer, including an initial dependence on androgen for growth followed by recurrence several months after castration (67,68). CWR22 tumor cells harbor a functional, mutated AR (H874Y) that displays broadened ligand specificity (69).

Prostate cancer cells may also evade androgen deprivation therapy through increased AR activation through binding of adrenal androgens and estrogens (69). Single amino acid point mutations, usually found in the ligand binding domain of AR, can decrease specificity of binding and increase AR transactivation of AR-regulated genes even in a testicular androgen depleted environment. The frequency of AR mutations increases after androgen ablation therapy and some suggest this may be due to a natural selection of these cells or environmentally directed mutations (70). Veldsholte et al. reported that a mutation in the AR ligand binding domain in LNCaP prostate cancer cells results in AR activation by non-androgen ligands, such as progesterone, estradiol, cyproterone, acetate, flutamide and nilutamide (65). Adrenal steroids

increasetransactivation of AR mutants as shown by Culig et al (71) and others (69,72-74). However, the low frequency of AR mutations cannot account for most instances or recurrence of prostate cancer during androgen deprivation therapy. The CWR22 human prostate xenograft contains an AR mutation at codon 874 (histidine replaced by tyrosine) which increases activation by adrenal androgens and hydroxyflutamide (69).

There are alternative mechanisms that may indirectly involve AR resulting in transactivation, such as coactivator binding or growth factor signaling. AR localizes in nuclei of prostate cancer cells despite low levels of circulating androgen and appears to mediate castration-recurrent growth after androgen deprivation (50). This could be explained by the presence of sufficient testosterone or DHT to activate AR in the microenvironment of castration-recurrent prostate cancer tissue (50,75,76). On the other hand, cell culture studies suggest that AR transcriptional activity involves growth factor signaling under conditions of androgen deprivation. Growth-factor activated pathways such as IGF-1, KGF and EGF can activate the AR and can induce AR target genes in the absence of androgen (77). Interleukin-6 (IL-6) is elevated in sera and tissue of patients with prostate cancer and is able to activate AR in a ligand-independent manner (78).

Studies by our group have shown that, in the presence of low levels of androgen, EGF increases AR transactivation through the phosphorylation of TIF2 (79) (Figure 1.2). We have also shown that heregulin (HRG) signaling through HER2 and HER3 (members of the EGFR1-4 tyrosine kinases) increases AR transactivation and alters growth in CWR-R1 cells (80) (Figure 1.3). Other studies have shown the involvement of receptor tyrosine kinase signaling by demonstrating that overexpression of Her2/neu can activate AR dependent genes in the absence of androgen but not in the absence of AR (79,80). Understanding the pathways of AR activation in response to mitogens as well as the role

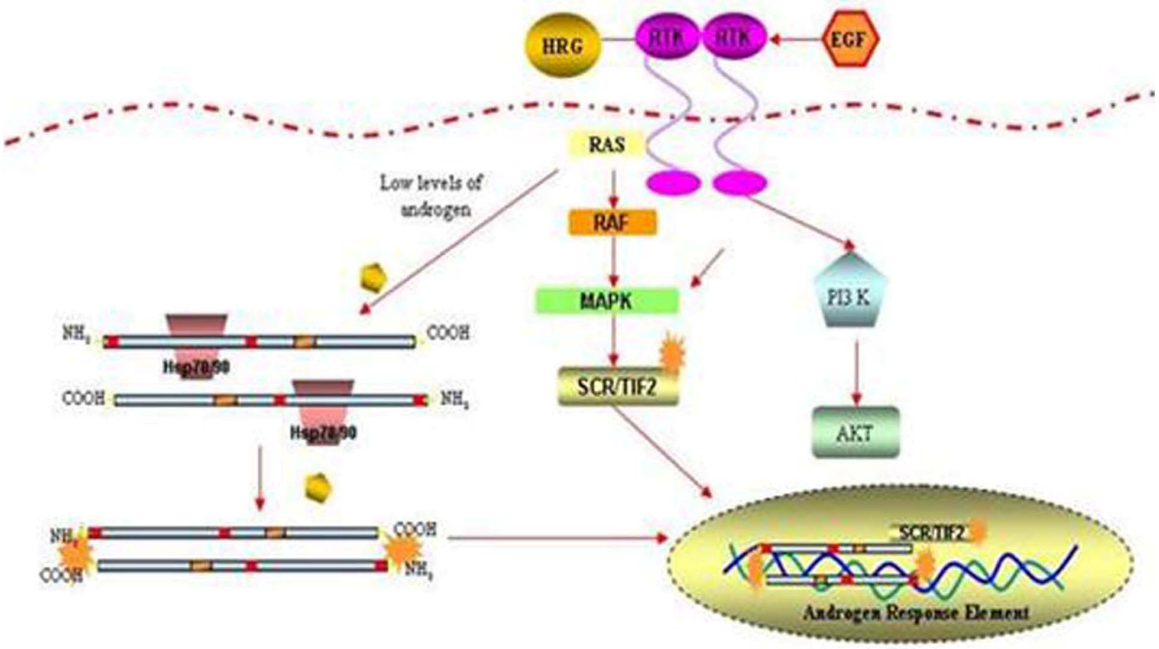

Figure 1.3. Convergence of growth factor–mediated pathways and the AR-signaling axis. Post-transcriptional modifications of coactivators in response to growth-mediated pathways lead to increased AR transactivation in the presence of low levels of androgen. The role of growth factor-mediated signaling on AR remains undefined**.**

of AR in evasion of hormone deprivation therapy is of critical importance given the substantial data that implicates its function during recurrence. Histone modifications influence binding of AR to cognate DNA sequences and can modulate the transcriptional activation of androgen-regulated genes. For instance, coactivator associated arginine methyl transferase (CARM1) mediates histone H3 methylation at androgen-regulated enhancer elements in response to androgenic stimuli (81). Interestingly, bicalutamide, an AR antagonist, was reported to facilitate histone methylation (82,83), suggesting that standard antiandrogen treatment may also work as a chromatin structure modifier.

Strategies directed to inhibit AR signaling after initial androgen ablation are aimed at decreasing the level of ligand by inhibiting adrenal androgen synthesis (ketoconazole) or inhibiting 17,20 lyase (abiraterone) and 5α-reductase (finasteride), important enzymes in the metabolism of androgens (84). Other agents for inhibiting AR function are antiandrogens based on AR crystal structures, antisense AR, or Hsp90 chaperone inhibitors that induce protein degradation. Histone deacetylase inhibitors and drugs targeting coregulatory molecules are also under evaluation as AR signaling blocking agents (84). Development of future therapy for prostate cancer will be aided by improving knowledge of AR action in prostate cancer.

ANDROGEN RECEPTOR PHOSPHORYLATION

Steroid receptors act as sensors for growth-factor signaling. AR acts as a transcription factor that integrates multiple extracellular signals (85). However, there is little consensus on the functional role of AR phosphorylation and its influence on transactivation, whether the effects are directly on AR or indirect through modifications

of associated coactivators. Interestingly, Akt was reported to suppress androgen-induced apoptosis by phosphorylating and inhibiting AR, suggesting AR has dual roles in the promotion of cell growth and apoptosis in response to posttranscriptional modifications (86).

Previous studies by our group and others have identified phosphorylation sites in AR (87,88). Recently, seven major AR phosphorylation sites were mapped, demonstrating constitutive phosphorylation of Ser-94, phosphorylation in response to androgen of serines 16, 81, 256, 308, 424 and 650, and phosphorylation in response to EGF, PMA and forsolkin of Ser-650 (85). Differential regulation of AR serine phosphorylation may explain the differences in AR activity induced by either androgens or growth factors. AR Ser-213 is phosphorylated *in vivo* specifically in non-proliferating epithelial cells (89). This suggests regulatory roles for AR phosphorylation in prostate cell growth and differentiation. Consensus phosphorylation sites in AR suggest that it can be a substrate for the DNA-dependent protein kinase, protein kinase A, protein kinase C, mitogen-activated kinase, and casein kinase II (90). The PKC isozyme family is comprised of at least 10 related kinases, key regulators of cellular responses that include proliferation, differentiation, transformation, survival, and apoptosis. These kinases are classified based on their structural and biochemical properties and each PKC has unique modes of regulation that involve phosphorylation, protein-protein interactions, and intracellular targeting—mechanisms that confer isozyme specificity in substrate phosphorylation (91-93) and some are downstreatm effectors of growth factor-induced stimuli. EGF increases AR activity in prostate cancer cell lines, suggesting that AR phosphorylation may result from growth factor signaling. Despite the large body of data implicating mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase

(PI3K) signaling on AR function, it remains to be established whether growth factor signaling directly phosphorylates AR. An *in vitro* study showed that mutating AR Ser-515 resulted in an unphosphrylated form of AR Ser-650 suggesting inter-site regulation of AR phosphorylation (94).

Tyrosine phosphorylation has also been implicated in AR transactivation. Prostate cancer growth was linked to src and activated Cdc42-associated tyrosine kinase (Ack1) mediated phosphorylation of AR tyrosine residues 267 and 363 in the AR NH_2-terminal region (40, 41). Further, the AR-Y534F mutation diminished Src-induced tyrosine phosphorylation, suggesting AR-Y534 is a site in mitogen-induced AR phosphorylation in prostate cancer cells (95).

Establishing the biological relevance of AR phosphorylation, including the effects on stability, nuclear localization, and expression levels is critical to our understanding of the convergence between androgen and receptor tyrosine kinase (RTK) activated pathways. Discerning the interaction between MAP kinase, PI3K signaling pathways and AR, may open possibilities for improved therapy.

GROWTH FACTOR SIGNALING IN ANDROGEN RECEPTOR TRANSCRIPTIONAL ACTIVITY

The epidermal growth factor (HER) family of RTKs, through binding of extracellular growth factor ligands mediate intracellular signal transduction. The HER family in humans is comprised of four members: ErbB1-EGFR, ErbB2 (HER2/neu), ErbB3/HER3, and ErbB4/HER4, which are activated upon ligand-induced receptor homo- and/or heterodimerization, suggesting that the HER receptor family has evolved a complex system of signal diversity. Activation of the EGF signaling network in response

to receptor tyrosine kinase activation results in a variety of responses, such as cell proliferation, differentiation, cell motility and survival (96). A wealth of clinical data has demonstrated that EGFR and HER2 contribute to the development of human cancer (97). However, it remains unclear how HER2/neu affects prostate cancer progression and AR function. Interestingly, EGF, the natural ligand for EGFR, induces AR transcriptional activity in the absence (98) and presence (99) of androgen. The implications of these observations for prostate cancer progression also remain ill-defined. We (preliminary results) observed a 2-3 fold increase in mouse mammary tumor virus (MMTV) reporter activity in response to EGF alone in CWR-R1 cells. The significance and mechanism of the EGF-mediated increase in transactivation and possible post-transcriptional modifications remain to be fully characterized and understood. Castration-recurrent CWR22 prostate xenograft tumors express high levels of EGF-related ligands compared with the androgen dependent tumor (76). We have shown that in CWR-R1 cells, androgen does not increase the expression of EGF (preliminary results), suggesting that there are paracrine or other signaling mechanisms independent of DHT that trigger growth factor cascades in the cell. Studies have not only implicated the EGFR axis in cancer progression and recurrence but the HER3 and HER4 axis as well. HER2/HER3 or HER2/HER4 heterodimerization occurs in response to HRG binding (80). We have demonstrated that HRG signaling through the HER2 and HER3 axis increases AR transactivation and the growth in CWR-R1 cells. Low level HER2/HER3, activation perhaps by an autocrine pathway, contributes to the proliferative signals during cancer recurrence (80). Studies using the dual EGFR/HER2 inhibitor PKI-166 suggest that signaling through the HER2/HER3 heterodimer stabilizes AR protein levels and optimizes binding of AR to promoter/enhancer regions of androgen-regulated genes

(100). Taken together, expression and signaling activity of growth factor receptors and their ligands in castration-recurrent prostate cancer suggest cross-talk between AR and loops which drives growth in the hormone deprived patient.

Residual androgens have been shown to be present in human prostate cancer tissue even after castration and, interestingly, it has been proposed that castration-recurrent prostate cancer cells, may synthesize testosterone from adrenal androgens or cholesterol (76). Regardless of the persisting presence of androgen in the microenviroment of cells, the influence of growth factor signaling on AR is important to understand the biology of prostate cancer in the androgen-deprived patient.

Growth factor stimulation of receptor tyrosine kinases activates different cell-signaling cascades that result in cell survival and proliferation. Nuclear hormone receptor function modulation implicated multiple kinase pathways (101). For instance, Kato et al. demonstrated that regulation of estrogen receptor α (ERα) by MAP kinase occurs directly through ERα phosphorylation (102). Others have shown that activation of the ERα can occur by p160 nuclear receptor coactivator binding (103,104). The serine/threonine kinase Raf-1 is a major regulator of the MAPK pathway and has been associated with the progression of prostate cancer to the more advanced disease (105). There is evidence to suggest that Raf/MEK/ERK signaling plays a critical role in the modulation of AR activity in response to Ras. For instance, MAP kinase activity in prostate cancer correlates with progression to advanced disease (106). MAP kinase signaling and overexpressed HER2 induce ligand-mediated increases in AR transactivation (107). Our observations linking EGF, MAP kinase, TIF2 and AR, suggest that kinase activity is important during recurrence in the CWR22 model. Bakin et al. (106) demonstrated that in prostate cancer cells expressing a constitutively active Ras, which up-regulates MAPK

activity, there is an increase in sensitivity to stimulation with low doses of androgen. Interestingly, other studies showed that increased pAkt (a second effector of Ras), alone or together with decreased pERK, is an important predictor of failure to reduce serum PSA levels (108). Thus, AR transactivation through the MAP kinase phosphorylation signal cascade may provide an alternative explanation to AR activation in ligand-depleted environments, although the mechanisms of kinase signaling modulation remain unclear. Additionally, protein kinase Akt is implicated in prostate cancer progression in an AR-dependent and AR-independent manner. Wen et al. showed that activation of the AR by HER2 is due to Akt phosphorylation at serine 213 and 791. HER-2/neu activated Akt (protein kinase B) to promotes prostate cancer cell survival and growth in the absence of androgen (109), implicating Akt signaling in prostate cancer recurrence. Further, prostate cancer invasion and metastasis are regulated partly by androgens through the PI3K pathway. Matrix metalloproteinase 2, which is highly expressed in aggressive prostate cancers and is involved in extracellular matrix degradation, is up-regulated by androgen through the PI3K pathway (106). Further, studies show that constitutively active Akt is involved in androgen-initiated up-regulation of hypoxia-inducible factor-1, which in turn stimulates vascular endothelial growth factor production, implicating PI3K signaling in the acquisition of aggressive metastatic potential of cancer cells (110). Activation of the PI3K pathway is implicated in antiapoptotic events by insulin-like growth factor protein I (IGFBP-1) through Akt (70). Activation of PI3K is counteracted by the phosphatase and tensin homolog PTEN. In prostate cancer, decreased PTEN activation is associated with high Gleason grade (111), and in LNCaP cells, loss of PTEN expression shows constitutively active Akt (112). Interestingly, PTEN is frequently mutated with loss of

function in advanced prostate cancers (113). Taken together, these data indicate that the Raf/MEK/ERK1/2 and PI3K-PDK-Akt pathways influence cancer progression.

ANDROGEN RECEPTOR DNA BINDING DOMAIN

Nuclear receptors are divided into four major subfamilies based on the DNA sequences they recognize (36,114). DNA binding domains of nuclear receptors contain three α-helices arranged in two zinc finger motifs (115). The first α-helix of the amino terminal zinc finger contains a 5 amino acid peptide residue stretch forming the proximal box (P-box) which makes base-specific contacts with the nucleotide bases in the DNA major groove and are responsible for the sequence-specific DNA recognition (116). AR is member of the class I receptor subgroup which includes the glucocorticoid (GR), progesterone (PR), and mineralocorticoid receptors (MR) all of which have a GSCKV P-box motif sequence that specifically recognizes the hexamer DNA motif 5'-TGTTCT-3' (114,117,118). Aminoacid residues that determine optimal spacing of three base pairs between core recognition motifs were localized to the region between the first and second cysteines of the second zinc finger module, called distal or dimerization box (D-box) based on the relative position and its role in receptor dimerization interface (116).

Base-specific interactions of P-box residues are stabilized by a number of nonspecific interactions of the amino- and carboxy-terminal part of the DBD and the DNA phosphate backbone (119). Class I NRs recognize similar DNA binding motifs and yet cellular responses are specific to androgen, glucocorticoid, mineralocorticoid, and progesterone stimulation. Some of the mechanisms postulated to explain differential receptor expression include hormone metabolism in hormone responsive tissue cells (120), differential cooperativity with transcription factor binding to hormone receptor-

specific regulatory DNA regions (121,122), and differential expression and/or receptor-specific interaction with co-regulators (123). AR binding to androgen response elements may lead to changes in the conformation of AR-NTD and intra-domain communication between the NTD and DBD of AR, resulting in changes in protein conformation and protein-DNA interactions (124). Multiple mechanisms likely establish specificity for AR-dependent DNA binding and target gene regulation.

Mutations in the P- and D-boxes of AR have been identified in the androgen insensitivity syndrome (AIS). One of these mutations at Ser-578 in the P-box resulted in partial responsiveness to androgen treatment. These cases indicate that the P- and D-boxes sites influence AR function during normal development and in hormone-related diseases.

Studies on PR suggest there is both basal and hormone induced phosphorylation and that hormone-induced phosphorylation of PR involves DNA-independent and DNA-dependent stages (125). A model of a three stage PR phosphorylation suggests phosphorylation occurs in a progressive cascade directly or by facilitating appropriate protein-protein contacts. Further investigation is required to determine the role of hormone-induced AR phosphorylation on DNA binding and the regulation of gene transcription.

The role of nuclear receptor DNA binding domains goes beyond DNA sequence recognition and binding. Nuclear export shuttling is regulated by signals within nuclear receptor DNA binding domains and nuclear localization signals. The GFP-AR-F582,583A double mutant resulted in AR nuclear arrest suggesting that residues of AR DBD influence receptor recycling (126). The mechanism by which AR DBD residues regulate nuclear exit are yet to be fully understood.

KU-70/80-MEDIATED NUCLEAR HORMONE RECEPTOR MODULATION

The DNA-dependent protein kinase (DNA-PK) has been described as a serine/threonine protein kinase that is activated upon association with DNA. DNA-PK is composed of a large catalytic subunit (DNA-PKcs) with a catalytic domain in the PI3K family (127), and a regulatory factor comprised of two tightly associated subunits of ~70 and 83 kD, referred to as Ku70 and Ku80, respectively (128). DNA-PK assists in repairing double-strand DNA breaks. The Ku-70/80 heterodimer is involved in later stages of the DNA damage response, particularly on non-homologous end-joining (129,130). The Ku70/80 heterodimer binds to the free DNA ends of a double strand break and recruits DNA-PKcs. Formation of the DNA-PK trimeric complex results in the recruitment and phosphorylation of multiple factors involved in the DNA repair and ligation (131). A second major role of DNA-PK involves regulation of V(D)J recombination as mutations of DNA-PKcs cause x-ray sensitive and V(D)J recombination-defective cells (132).

Activation of DNA-PK involves interactions with DNA and other proteins. Recruitment of DNA-PKcs to Ku facilitates the interaction of DNA-PKcs with DNA, releasing its catalytic subunit through conformational changes (128). While DNA alone can activate DNA-PKcs in the absence of Ku (133), protein–protein interactions may also regulate DNA-PK activity. DNA-bound C1D, a human DNA binding protein, can activate DNA-PK in a DNA end-independent manner, by altering the structure of the DNA double helix (134). Together, the data suggest there are multiple mechanisms of DNA-PK activation which may or may not involve canonical double strand DNA break stimuli.

The DNA-PK has been implicated in a number of regulatory processes in mammalian systems, such as transcriptional regulation. Ku-70/80 is localized to the promoter of androgen-dependent PSA gene, as shown by chromatin immunoprecipitation. A report suggest Ku-70/80 act as AR transcriptional coactivators in reporter assays as siRNA-induced knockdown of Ku-70/80 caused a modest reduction in androgen-dependent PSA transcriptional activation (135). However, a second report demonstrated increased AR-dependent transcription of several androgen-regulated genes in response to chemical inhibition of DNA-PK catalytic activity (136). There is little consensus on the role and mechanisms of DNA-PK-mediated regulation on AR activity. DNA-PK has been shown to interact with and/or phosphorylate a number of transcription factors including EGFR (137), transcription factor IID (TFIID) (138), Oct-1 (139), PR (140) and GR (141,142). Early studies on PR phosphorylation using HeLa nuclear extracts suggested PR phosphorylation by DNA-PK in response to addition of DNA harboring a progesterone-response element, suggesting that at least one mechanism of transcriptional regulation by DNA-PK is through phosphorylation of nuclear hormone receptors (143). DNA-PK phosphorylates target proteins preferentially, although not exclusively, at the consensus Ser/Thr-Gln (144), however there is no evidence to date of DNA-PK phosphorylation of AR *in vivo*.

SELF-COMPLEMENTARY ADENO-ASSOCIATED VIRAL VECTOR-MEDIATED INHIBITION OF PROTEIN EXPRESSION

The emergence of RNA interference has enabled the selective suppression of gene expression in mammalian cells. Small inhibiting RNAs (siRNAs) trigger specific catalytic degradation of complementary mRNAs through the formation of multicatalytic

complexes that minimize cytotoxicity associated with long antisense RNAs (145,146). Long-term targeted gene suppression through siRNA can be attained in mammalian cells using RNA polymerase III promoter-driven hairpin siRNA-producing vector-based cassettes. The adeno-associated viral vector delivery system (AAV) has been widely used for transgene delivery in mammalian cells. Self-complementatry AAV (scAAV) is a derivative of the conventional recombinant AAV which was recently developed as an enhanced-efficiency gene delivery vector (147).

Conventional single-stranded recombinant AAV (rAAV) requires second strand synthesis before genes can be expressed. scAAV bypasses this rate limiting step by delivering a duplex genome. This was achieved by deleting a minor portion (28 of 145 bases) of one of the terminal repeats (TR) eliminating it as a replication origin yet forming a wild-type AAV TR hairpin structure (148). Rolling hairpin replication from the remaining wild-type terminal repeat creates single stranded, dimeric inverted repeat genomes, with the altered terminal repeat sequence situated in the middle of the molecule and a wild-type TR at each end (149). This unique structure allows for intramolecular base pairing to generate the double stranded DNA template for gene expression in the absence of DNA synthesis in the target cell. To date, no scAAV driving expression of ARsiRNA have been published. Vectors that provide high and potent transduction efficiency to inhibit AR protein are valuable tools for the study of AR in prostate cancer recurrence.

DISSERTATION RESEARCH OBJECTIVES

AR and mitogen signaling are increased during prostate cancer recurrence. The relative lack of consensus on the functional effects of AR phosphorylation leave

unanswered the question of the significance of growth factors during disease progression and the mechanisms by which, during androgen deprivation, kinases regulate AR function. The objectives of this dissertation research were to develop a tool for the inhibition of AR in prostate cancer cells in order to determine the role of AR in castration- recurrent prostate cancer growth. A further goal was to identify EGF-mediated AR phosphorylation sites that contribute to AR transactivation in the absence of androgen, and the mechanisms by which mitogen signaling influences AR function, sub-cellular localization and protein-protein interactions.

EXPERIMENTAL PROCEDURES

CELL CULTURE MEDIUM

CWR-R1 cells were maintained either in Richter's improved minimal essential prostate growth medium (Irvine Scientific, Santa Ana, CA) or Dulbecco's modified Eagle medium (DMEM, Gibco, Carlsbad, CA), each supplemented with 10 nM nicotinamide, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 100 units/ml penicillin, 100 µg/ml streptomycin and 2% fetal bovine serum (FBS). DMEM growth medium was further supplemented with 15 mM HEPES, pH 7.2. LNCaP cells were maintained in RPMI 1640 medium with 10% fetal bovine serum. LNCaP-C4-2 cells were grown in T media (DMEM:Ham's F-12 with 5% fetal bovine serum, 5 µg/ml insulin, 13.65 pg/ml triiodothyronine, 5 µg/ml apo-transferrin, 0.244 µg/ml d-biotin, and 25 µg/ml adenine. LAPC-4 cells were cultured in RPMI-1640, 10% FBS, 100 units/ml penicillin, 200 mM L-glutamine, and 1nm R1881. XR-V15B cells were cultured in Ham's F10 with 2 mM L-glut, 10% FBS and 100 units/ml penicillin. Monkey kidney (COS-1) cells were cultured in DMEM-H, 10% bovine calf serum, 100 units/ml penicillin, 200 mM L-glutamine and 15 mM HEPES. Human endometrial Ishikawa cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin / streptomycin, and 2 mM L-glutamine.

RNA INTERFERENCE AND TRANSCRIPTION ASSAYS

Duplex AR-siRNA-3 was ucaaggaacucgaucguauuu sense and auacgaucgaguuccuugauu antisense sequence, and AR-siRNA-4 was gaaaugauugcacuauugauu sense and ucaauagugcaaucauuucuu antisense sequence (NCBI M20132) (SMART selection designed siRNAs, Dharmacon Inc., Lafayette, CO). The control was siCONTROL nontargeting siRNA pool (Dharmacon Inc.). CWR-R1 prostate cancer cells derived from the CWR22 castration-recurrent prostate cancer xenograft (6, 16) were transfected using Effectene (Qiagen, Valencia, CA) with 10 nM AR siRNA or control siRNA duplex and 0.1 μg mouse mammary tumor virus luciferase reporter (MMTV-Luc). CWR-R1 cells were plated (1.6 x 10^5 cells/well) in 12 well plates using Richter's improved minimal essential prostate growth medium (Irvine Scientific, Santa Ana, CA) or Dulbecco's modified Eagle medium (DMEM, Gibco, Carlsbad, CA), each supplemented with 10 nM nicotinamide, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 100 units/ml penicillin, 100 μg/ml streptomycin and 2% fetal bovine serum (FBS). DMEM growth medium was further supplemented with 15 mM HEPES, pH 7.2. DNA and siRNA (220 μl) containing per well, 45 μl EC buffer (Qiagen), 1 μl enhancer, 1 μl Effectene reagent (Qiagen) and 200 μl 2% serum containing medium was added to cell cultures containing 0.8 ml fresh medium. The next day the medium was replaced with phenol red-free, serum-free Improved Minimal Essential Zinc Option medium (Gibco/Invitrogen) with and without DHT and EGF and incubations were continued for 24 h. Human endometrial Ishikawa cells were transfected using FuGENE-6 (Roche Applied Science) as previously described (17) using 0.025 μg pCMV-AR and 0.1 μl

PSA-Enh-Luc. Cells were harvested in 0.25 ml lysis buffer containing 1% Triton-X-100, 2 mM EDTA and 25 mM Tris-phosphate, pH 7.8 (18). Luciferase activity was determined using an automated LumiStar Galaxy multiwell plate reader luminometer (BMG Labtechnologies, Durham, NC). Transfection data are representative of at least three independent experiments.

XRV15B TRANSIENT TRANSFECTION

XRV15B cells were transfected using calcium phosphate DNA precipitation as described elsewhere (150). XRV15B cells ($2.5x10^6$ cells/6-cm dish) were transfected with 1 µg pCMV-AR in the absence and presence of 4 µg pCDNA-Ku80, and 4 µg MMTV-Luc wild-type (wt), Δ-381:-394, or Δ-421:-364 as indicated. After transfection, XRV15B cells were placed in serum-free, phenol red-free medium in the absence and presence of 1 nM DHT. The next day, the media with and without DHT were exchanged, and 24 h later luciferase activity was measured.

CELL GROWTH ASSAYS

CWR-R1 prostate cancer cells (1.6×10^5/well) in 12-well plates were allowed to grow in prostate growth medium for 24 h. Cells were infected with 10^3 viral particles/cell in medium containing 2% FBS by rocking for 30 min at room temperature, and incubated overnight at 37˚C in serum containing medium. Cells were transferred to serum-free, phenol-red free medium in the absence and presence of DHT and EGF. Duplicate wells were treated and assayed on days 1, 3 and 5 after infection. One-tenth volume of 2- (2-methoxy- 4- nitrophenyl)- 3- (4- nitrophenyl)- 5- (2, 4- disulfo- phenyl)- 2H- tetrazolium

monosodium salt (WST-8, Dojindo Molecular Technologies, Gaithersburg, MD) was added to the wells, incubated for 2.5 h at 37˚C and absorbance determined at 450 nm using a plate reader. The effect of inhibiting protein kinase C (PKC) on cell proliferation was assayed using cells plated in medium containing 2% FBS. The next day, cells were treated with calphostin (Calbiochem, La Jolla, CA), a PKC inhibitor, in the absence and presence of EGF. Proliferation assays were performed 1, 3, 5 and 7 days after treatment (Figure 2.1)

MOLECULAR CLONING AND CHARACTERIZATION OF SCAAV VECTORS

pSilencer 1.0-U6 siRNA containing an RNA polymerase III promoter (14, 19) (Ambion Inc., Austin, TX) and the Insert Design Tool for the pSilencer Vectors (Ambion) were used to generate hairpin siRNA encoding DNA oligonucleotide sequences based on the siRNA-3 sequence (Figure 2.2). A central loop sequence ttcaagaga and single overhang strand were added for cloning. Oligonucleotides that target AR were aaggaactcgatcgtatcattcaagagatgatacgatcgagttccttgatttttt sense (55 nucleotides) and aattaaaaaatcaaggaactcgatcgtatcatctcttgaatgatacgatcgagttccttggcc antisense sequence (63 nucleotides) with 5´ EcoRI and 3´ ApaI terminal restriction sites. Control scAAV duplex oligonucleotides were ttctccgaacgtgtcacgtttcaagagaacgtgacacgttcggagaatttttt sense (53 nucleotides) and aattaaaaaattctccgaacgtgtcacgttctcttgaaacgtgacacgttcggagaaggcc antisense sequence (61 nucleotides). Oligonucleotides were annealed by incubating at 90ºC for 3 min in the presence of 46 µl

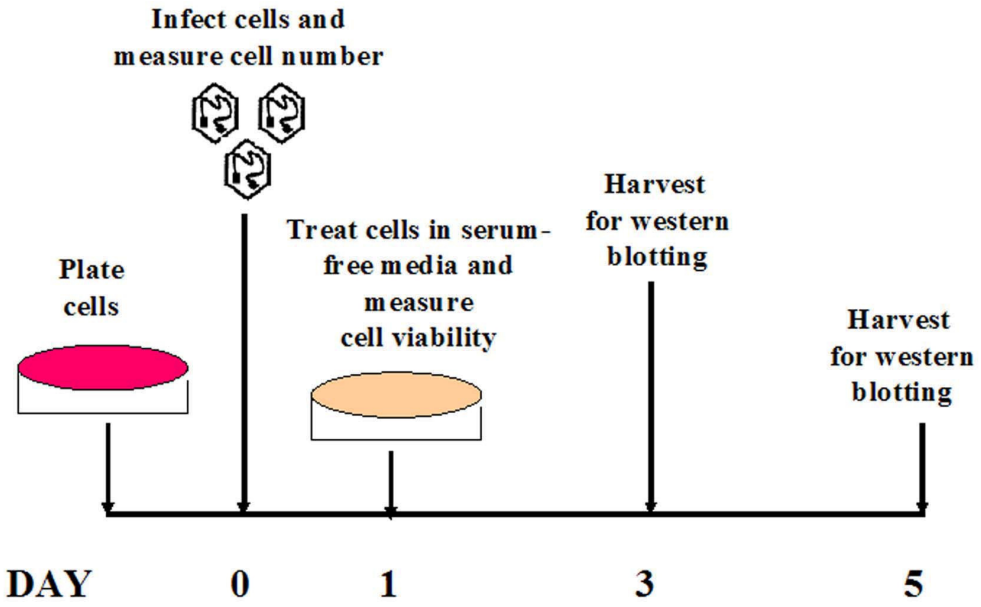

Figure 2.1. scAAV-mediated AR inhibition and its effects on CWR-R1 growth: Study Design. To test the effects of scAAV transduction of AR or control (CTR) siRNA on CRW-R1 cell growth, experiments were designed to test cell viability 1, 3 and 5 days after infection. 24h after infection cells were treated with and without DHT and EGF in serum-free, phenol red-free medium. Western blots were performed 2 and 5 days after infection to determine AR protein levels.

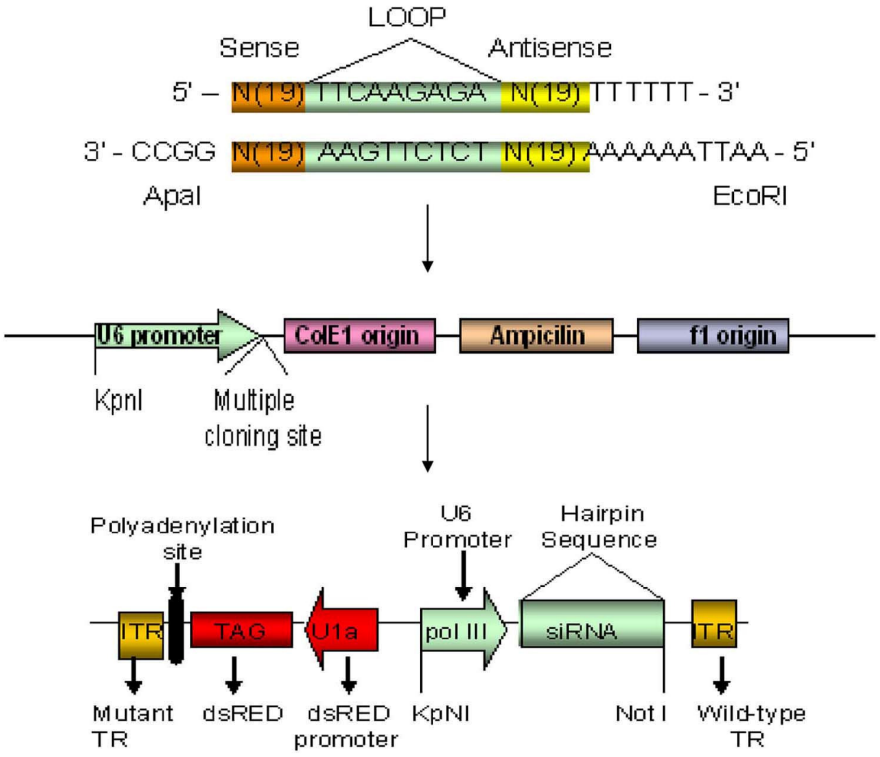

Figure 2.2. Cloning of the siRNA targeting sequence into scAAVplasmid. The AR siRNA targeting sequence was entered into a web-based converter that generated the sense and antisense oligonucleotides necessary to transcribe the silencing hairpin. The oligos were annealed and ligated into the pSilencer vector using the ApaI and EcoRI enzyme sites located in the multiple cloning site downstream of the U6 promoter. Cloning of the AR siRNA and control siRNA oligonucleotides was confirmed by automated DNA sequencing. KpnI and NotI were utilized to clone the U6 promoter and DNA targeting hairpin into the genome of the scAAV (p-hpa-trs-SKAAV2). The scAAV type II contains a fluorescent tag (dsRED) driven by the U1a promoter, a polyadenylation site, a mutant and wild type inverted terminal repeats (ITRs) and the polymerase III promoter (U6) upstream of the cloning region for the haipin siRNA.

annealing buffer containing 100 mM K-acetate, 2 mM sodium acetate and 0.03 M HEPES, pH 7.4, followed by 1 h incubation at 37ºC. pSilencer 1.0-U6 was linearized with ApaI and EcoRI and ligated overnight at 25ºC to the siRNA insert. The type II scAAV expression vectors ptrs-U1a-RFP-U6 and ptrs-U1a-green fluorescent protein and helper plasmids pXX6 (adenoviral helper genes) and pXX2 (AAV helper genes) were generously provided by Douglas M. McCarthy and Jude R. Samulski (Gene Therapy Center, University of North Carolina at Chapel Hill). p-trs-U1a-RFP-U6 contains a small nuclear RNA U1a promoter to drive expression of red fluorescent protein derived from pDSRED2-C1 (Clontech, Palo Alto, CA). The U6 promoter and duplex sequences were excised from pSilencer 1.0-U6 and cloned into ptrs-U1a-RFP-U6 linearized with NotI and KpnI. In frame ligation was confirmed by sequencing. scAAV vectors produced in human embryonic kidney 293 cells using three-plasmid transfection were purified as described previously (20).

Human embryonic kidney 293 cells (2 x 10^7/dish) in DMEM containing 10% FBS were plated in twenty 15 cm dishes for 70-80% confluency after 24 h. Each plate was passaged 1:4 in 15 cm dishes and 16 h later, the medium was replaced with complete Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA) containing 10% FBS, and incubated for 3 h before transfection. pXX6 helper plasmid (90 μg) was combined with 30 μg control or AR-siRNA-scAAV vector plasmid, 30 μg pXX2 helper plasmid, 0.25 M $CaCl_2$, 0.25 M NaCl, 1.5 mM sodium phosphate and 0.05 M HEPES, pH 7.2. Cells were incubated for 24 h and medium was replaced with DMEM containing 2% FBS. Virus was collected 48 h post-transfection by lysing cells using 3 freeze-thaw cycles. scAAV vectors were purified by isopycnic centrifugation in CsCl (ρ 1.4 g/ml) in an SW41 rotor (Beckman, Palo Alto, CA) at 40,000 rpm for 48 h at 10ºC. Fractions were

collected, semi-quantitated by slot-blot and peak fractions pooled and dialyzed in phosphate buffered saline (PBS) overnight at 4ºC (Figure 2.3). To test transduction efficiency of the scAAV serotype II in CWR-R1 cells, cells were infected with a scAAV type II ptrs-U1a-green fluorescent protein virus in medium containing 2% FBS. Cells were harvested 1 and 9 days post-infection. Green fluorescent protein expression in transduced cells was analyzed by fluorescence-activated cell sorting using a FACScan1 cytometer (Becton-Dickinson). Forward and side scatter parameters were set according to cell size, and the setting for fluorochrome detection adjusted so that fluorescence intensity of uninfected negative control cells was within the first decade of the 4 scale log plot.

SCAAV INFECTION OF PROSTATE CANCER CELL LINES

CWR-R1 prostate cancer cells (1.6 x 10^5/well) in 12-well plates were allowed to grow in prostate growth medium for 24 h. Cells were infected with 10^3 viral particles/cell in medium containing 2% FBS by rocking for 30 min at room temperature (unless indicated otherwise) and incubated overnight at 37˚C in serum containing medium. Cells were transferred to serum free, phenol-red free medium in the absence and presence of DHT and EGF. Duplicate wells were treated and assayed on days 1, 3 and 5 after infection. One-tenth volume of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium monosodium salt (WST-8, Dojindo Molecular Technologies, Gaithersburg, MD) was added to the wells, incubated for 2.5 h and absorbance determined at 450 nm using a plate reader. The effect of inhibiting protein kinase C (PKC) on cell proliferation was assayed using cells plated in medium containing

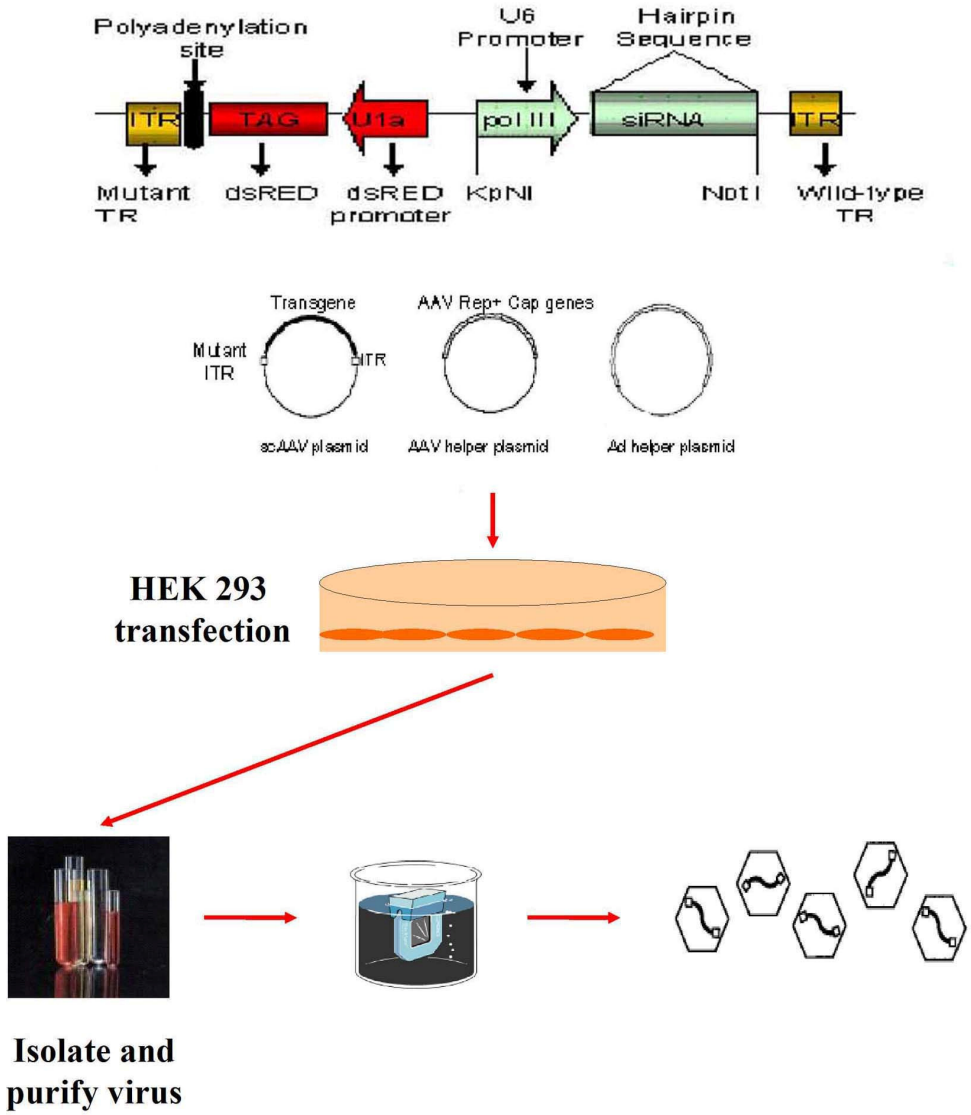

Figure 2.3. Generation and quantification of the AR siRNA and control scAAVs. A three-plasmid system was used to generate scAAV verctors. $5x10^8$ HEK293 cells/P150 plate were transfected with 360 µg of Ad helper plasmid (pxx6), 120 µg AAV helper plasmid (pxx8) and 120 µg of the scAAV plasmid using the $CaPO_4$ procedure in 10% FBS media. 24 h after transfection cells were changed to 2% FBS media and cells were incubated for 24 more hours. Cells were then collected, lysed by freeze-thaw cycles, sonicated, and the virus precipitated with saturated ammonium sulfate. A CsCl gradient purification was performed for 48 h. Viral fractions were collected and a slot-blot performed. Standard amounts (indicated above the lines) of vector DNA (ng) were transferred using a slot-blot apparatus to a nitrocellulose membrane. The DNA was crosslinked and the blot probed with radioactively labeled linearized vector for 2 h. Virus prep #1 and #2 represent the scAAV DNA viral titer.

2% FBS. The next day, cells were treated with Calphostin (Calbiochem, La Jolla, CA), a PKC inhibitor, in the absence and presence of EGF. Proliferation assays were performed 1, 3, 5 and 7 days after treatment.

PLASMIDS AND SITE-DIRECTED MUTAGENESIS

Expression vectors pCMVhAR for full-length human AR (21), pCMVhAR-(1-660) for the AR NH_2-terminal, DNA binding and hinge regions, and AR-(507-660) for part of the AR NH_2-terminal domain with DNA binding domain and hinge region, were described previously (1, 22). Mutations in pCMVhAR were generated by PCR amplification and verified by sequencing. Flag-AR-(507-660) was created by subcloning pCMVhAR mutants into EcoRI and SalI digested pCMV-Flagb. Glutathione-S-transferase (GST) fusion vector GST-AR-(1-660) was prepared as described previously (23). GST-AR-(1-660)-S578A was generated by site directed mutagenesis using the QuikChange kit (Stratagene) and confirmed by sequencing. Prostate specific antigen enhancer luciferase reporter vector (PSA-Enh-Luc) was provided by Michael Carey (University of California, Los Angeles) and contains the PSA upstream enhancer region (24). MMTV-Luc was provided by Stanley M. Hollenberg and Ron M. Evans (Salk Institute).

NORTHERN BLOTTING

CWR-R1 cells (5 x 10^6/10-cm dish) were plated in prostate growth medium lacking EGF. The next day, cells were washed with phosphate-buffered saline, and the medium was changed to phenol red-free improved MEM zinc option (Invitrogen)

containing 0.2% albumin (AlbuMax I; Invitrogen). After an overnight incubation, cells were treated without or with 100 ng/ml EGF for 24 h in the same medium prior to RNA isolation using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. RNA (15 µg) aliquots were fractionated on 1% agarose gels, transferred to nylon membranes, and hybridized with ^{32}P-labeled pSG5TIF2 BamHI fragment containing nucleotides 629-869 and a pGEM-18S-4 (Promega) Sp6-generated fragment for control 18 S ribosomal RNA.

REAL-TIME REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

$1.6x10^6$ LacZ- or Δ-TR-transduced CWR-R1 cells were plated in prostate growth medium lacking EGF, serum-starved for 24 h and treated with or without 0.1nM DHT. To determine EGF mRNA levels in CWR-R1 cells, one day after plating, cells were serum starved for 24 h and the next day treated for 2, 5, 8 or 24 h with or without 10 ng/ml EGF. For EGF mRNA expression studies, $1.6x10^6$ CWR-R1 cells were plated, serum starved for 24 h and treated the next day with 0.1, 1 or 10 nM DHT. Cells were washed in PBS, harvested and mRNA extracted and purified (RNeasy® Midi Kit, Quiagen, Valencia, CA). RNA quality was verified (Agilent LabChip Bioanalyzer, UNC-CH Genomics Core) and quantitative one-step-reverse transcriptase (RT)-realtime PCR was performed (Light Cycler System, Roche). The housekeeping gene human β-glucuronidase (GusB) was used to perform relative quantification. Standard curves for GusB, hK2, and EGF transcription were generated and efficiencies were calculated to validate GusB as a suitable housekeeping gene for data normalization. The following primer and probe sequences were used: hK2 forward 5'-

GCCTTAGACCAGATGAAGACTCCA-3'; hK2 Reverse, 5'-CCCAGGACCTTCACAACATC-3'; hK2 Probe, 5'-6-FAM-TGACCTCATGCTGCTCCGCCTGT-BHQ-1-3' (81). GUSB Forward, 5'-GGTGCTGAGGATTGGCA-3'; GusB Reverse 5'-TAGCGTGTCGACCCCATTC-3'; GUSB probe, 5'-6-FAM-TGCCCATTCCTATGCCATCGTGTG-TAMRA-3' (GUSB sequences kindly provided by Dr. Sergio Onate, Roswell Park Cancer Institute); EGF Forward, 5'-CTGTACTCTTGGGTGT-3'; EGF Reverse 5'-AGCAATCACATTCCCAGGAT-3'; EGF Probe 5'-6-FAM-AGGATTTGTTCTGCTTCCTGATGGGA-TAMRA-3' (151). Samples were run in duplicate and the hK2/GUSB ratio was used to compare the effects of DHT on hK2 transcription in two independent experiments.

IN VITRO KINASE ASSAYS

GST-AR-(1-660) and GST-AR-(1-660)-S578A were expressed in BL21 *Escherichia coli* cells treated with 1 mM isopropyl-β-thiogalactoside for 24 h at 16ºC during log phase growth. Glutathione-agarose beads (Amersham, Pharmacia Biotech Piscataway, NJ) were incubated for 1 h at 4ºC with sonicated bacterial supernatants containing GST-AR fusion proteins. Beads were washed 3 times with PBS containing 1% Triton-X-100, followed by 3 washes with kinase buffer containing 10 mM EGTA, 0.1 M $MgCl_2$ and 0.4 M 2-(N-morpholino) ethanesulfonic acid, pH 6.0. Part of the sample eluted with sample buffer was analyzed on 8–12% acrylamide gradient gels containing SDS. A serial dilution of bovine serum albumin was analyzed in parallel to estimate protein recovery. Bound protein (10–15 μg) was assayed for PKC phosphorylation. GST

beads were resuspended in 30 µl kinase buffer containing 10 or 100 µCi [γ-32P] adenosine triphosphate (3000 Ci/mmol) and 20 ng purified PKC (catalytic subunit, rat brain, Calbiochem, San Diego, CA) in the absence and presence of 1 µM unlabeled adenosine triphosphate and either 2.5 µg histone-H1 or 2.5 µg wild-type or mutant GST-AR-(1-660) fusion protein. After 10 min at 30ºC, reaction products were resolved on 8–12% acrylamide gradient gels containing SDS, which were dried and analyzed by autoradiography. To verify equal loading, gels were rehydrated in PBS and stained with Coomassie blue or half of the input resin was resuspended in 2X SDS sample buffer and analyzed by immunoblotting using AR52 antibody. Autoradiographs were quantitated by densitometric scanning using Image-Pro Analyzer Software (Media Cybernetics, Inc., Bethesda, MD).

WESTERN BLOTTING AND IMMUNOPRECIPITATION

AR phospho-Ser-578 antipeptide antibody was raised in rabbits (21st Century Bio, Marlboro, MA) by immunizing with two bovine serum albumin-coupled peptides that were based on human AR sequence ^{572}GALTCGSCKVFFKRA^{586}. Peptide 1 was acetyl-G[pS]-aminobutyrate-KVFFKRA-amino-hexanoic acid-C-amide. Peptide 2 was acetyl-C-aminohexanoic acid-GALT-aminobutyrate-G[pS]-aminobutyrate-KVFF KRA-amide. Nonphospho-peptide acetyl-C-amino-hexanoic acid-GALT-amino-butyrate-GS-amino-butyrate-KVFFKRA-amide was used to establish binding specificity. Aminobutyrate replaced cysteine to avoid disulfide bonding, aminohexanoic acid was a 6 carbon spacer, and the carboxyl-terminal cysteine was coupled to bovine serum albumin.

Immunoblotting was performed using monkey kidney COS-1 cells maintained in DMEM containing 2 mM L-glutamine, 10% bovine calf serum, penicillin, streptomycin and 20 mM HEPES, pH 7.2. COS cells (2.5 x 10^6/10 cm dish) were transfected with 2 μg DNA using DEAE dextran (25). Cells were treated 24 h after transfection with and without 10 ng/ml EGF for 5 h. For MAP kinase inhibition studies, cells were treated for 1 h before transfection with and without U0126 (Promega). The next day cells were serum-starved in the absence and presence of U0126 and media replaced 24 h later with and without 10 ng/ml EGF for 5 h. Cells were rinsed with PBS, scraped into 1.5 ml cold PBS and centrifuged at 12,000xg for 2 min. The buffer was aspirated and cells were resuspended and vortexed for 10 sec in 50–100 μl buffer containing 1% Nonidet P-40, 0.15 M NaCl, 1% sodium deoxycholate, 0.1% SDS, 0.5 mM EDTA, 50 mM Tris-HCl, pH 7.5, 0.02 mg/ml pancreas extract, 1 mM phenylmethylsulfonyl fluoride, 0.005 mg/ml pronase, 0.0005 mg/ml thermolysin, 0.003 mg/ml chymotrypsin and 0.33 mg/ml papain (Roche Applied Science). After a 15 min incubation on ice, lysates were centrifuged for 15 min at 20,000xg and protein concentrations determined using the Bio-Rad assay. Protein (25 μg) was incubated with and without 2.5 units λ-phosphatase (Sigma) in phosphatase buffer (Sigma) containing 2 mM $MnCl_2$. To inhibit λ-phosphatase activity, lysates were incubated with 2.5 mM sodium vanadate, 10 mM sodium fluoride and phosphatase Cocktail Inhibitors 1/2 (Sigma) for 30 min at 25ºC.

To express Flag-tagged constructs in CWR-R1 cells, 3 μg DNA/5 x 10^6 cells/10 cm dish was transfected using Effectene (Qiagen). The next day CWR-R1 cells were treated with and without 10 ng/ml EGF for 5 h, harvested in cold PBS from 5 pooled dishes for each treatment group and lysed in immunoprecipitation buffer containing 0.5% Nonidet P-40, 10% glycerol, 0.05 M sodium fluoride, 0.15 M NaCl, 50 mM Tris-HCl, pH

7.6, and phosphatase and protease inhibitors. For immunoprecipitation of full-length AR expressed in COS cells, 0.5 µM DHT was added to the lysis buffer. Approximately 1 mg protein from two 10 cm COS cell dishes was precleared using Sepharose CL-4B (Sigma) and immunoprecipitated using anti-FLAG M2 Affinity Resin (Sigma). The final pellets were washed 3 times with immunoprecipitation buffer with and without 0.5 µM DHT and resuspended in 60 µl 2X sample buffer containing 3.3% SDS, 10% glycerol, 0.2% 2-mercaptoethanol and 20 mM Tris-HCl, pH 6.8 and analyzed by immunoblot. For immunoblots of cell extracts, 4% total protein was separated on 10% acrylamide gels containing SDS. After electrophoresis, gel proteins were electroblotted to Immobilon-P membrane (Millipore Corp., Bedford, MA) overnight. Transfer blots were blocked overnight at 4ºC in 5% milk, 0.9% NaCl, 0.05% Tween-20 and 0.01 M Tris-HCl, pH 7.5. Blots were incubated for 1–2 h with 2.5 µg/ml rabbit polyclonal AR52 antibody targeting human AR NH_2-terminal amino acid residues 544 to 558 (21), anti-Flag M2 monoclonal antibody (1:2000 dilution, F-3165 Sigma), ß-actin antibody AC-15 (1:5000 dilution, Abcam, Inc.) or Ku (p70) and Ku (p80) antibodies (0.5 µg/ml, MS-329 and MS-285, Lab Vision Corporation, Fremont, CA). Anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary IgG antibodies (1:10,000 dilution, Amersham Life Sciences) were incubated for 30 min at room temperature. Signals were detected using chemiluminescence (SuperSignal West Dura Extended Duration Substrate, Pierce, Rockford, IL).

Reactivity of AR phospho-Ser-578 antisera was determined by immunoblot of BSA, BSA-coupled AR-(572-586) non-phosphorylated peptide and AR-(572-586) phospho-Ser-578 peptides 1 and 2 immunogens, and wild-type Flag-AR-(507-660) and the S578A mutant. COS cells (2.5 x 10^6/10 cm dish) were transfected with 2 µg wild-type

Flag-AR-(507-660) and the S578A mutant, and 24 h later cells were placed in serum-free, phenol-red free media for 24 h, and treated with and without 10 ng/ml EGF for 5 h. Cells were lysed in immunoprecipitation buffer containing phosphatase and protease inhibitors and immunoprecipitated using anti-FLAG M2 affinity resin (Sigma) and resolved on a 12% acrylamide gel containing SDS. Transfer blots were probed with AR phospho-Ser-578 antisera (1:100 dilution) and 2.5 µg/ml AR52 antibody at room temperature for 2 h and with secondary antibody as described above. After chemiluminescence detection, membranes were rinsed with distilled water and incubated for 10 min at room temperature with 0.2% Ponceau-S in 0.1% glacial acetic acid.

IMMUNOCYTOCHEMISTRY

Immunocytochemistry was performed in COS cells (1×10^5 cells/well) in 12 well plates with a coverglass (26). Cells were transfected using Effectene with 0.2 µg wild-type Flag-AR-(507-660) or the S578A mutant and serum starved 24 h. Cells were fixed for 10 min at room temperature with 4% paraformaldehyde in PBS, permeabilized for 5 min at 4°C with 0.2% Triton-X-100 in PBS, blocked for 1 h at room temperature with 0.5% bovine serum albumin in PBS (13), and incubated for 1 h in 0.5% bovine serum albumin containing AR52 antibody (2.5 µg/ml) and for 30 min at room temperature with fluorescent-(FITC)-conjugated anti-rabbit secondary antibody (1:75 dilution, Jackson ImmunoResearch Laboratories, Inc.). Slides were viewed using an Olympus BX60 microscope with original magnification of 40X.

NUCLEAR/CYTOPLASMIC EXTRACTION

Flag-AR fusion vectors were transfected into COS cells (2.5 x 10^6/10 cm dish) using DEAE dextran. The next day cells were serum starved and 24 h later treated with 10 ng/ml EGF for 5 h. Nuclear extracts were prepared using the Nuclear Extract Kit (Active Motif, Carlsbad, CA). Cells were washed with ice-cold PBS containing phosphatase inhibitors, scraped and pelleted for 5 min at 2600xg, resuspended in 550 µl hypotonic buffer and incubated 15 min on ice. Detergent (25 µl) was added and samples were vortexed 10 sec at the highest setting. Cell suspensions were centrifuged for 30 sec at 14,000xg and cytoplasmic extracts were stored at -80ºC. Nuclear cell pellets were resuspended in 50 µl complete lysis buffer containing 1 mM DTT and protease inhibitor cocktail. Lysates were vortexed for 10 sec and suspensions incubated for 30 min on ice on a rocking platform at 4ºC. Extracts were vortexed 30 sec, centrifuged 10 min at 14,000xg and nuclear fractions transferred to pre-chilled microcentrifuge tubes. Protein concentration was determined and nuclear and cytoplasmic extracts analyzed by immunoblotting using 2.5 µg/ml AR52, tubulin-α (2 µg/ml, Thermo Fisher scientific, Fremont, CA) and Laminin B1 antibodies (2 µg/ml, Active Motif, Carlsbad, CA).

RESULTS

ANALYSIS OF EGF REGULATION OF TIF2 MRNA IN CWR-R1 CELLS

We showed previously that progression to recurrent growth of prostate cancer is associated with increased levels of p160 coactivators (49,79) that increase AR activity (48). We also demonstrated that steady state levels of TIF2 protein were highest in the CWR-R1 cell line compared with PC3, LNCaP, and LNCaP-C4-2 prostate cancer cell lines, and HeLa cells (48). TIF2 was undetectable in a nontransformed human foreskin fibroblast cell line and lower levels of TIF2 were detected in COS and CV1 cells compared to the prostate cancer cell lines. To test whether the EGF-mediated increase in AR protein was at the level of transcriptional regulation, we performed northern blot analysis in CWR-R1 cells. We ruled out a direct effect of EGF on TIF2 transcription, since northern blot analysis showed similar levels of TIF2 mRNA in CWR-R1 cells with and without EGF treatment (79) (Fig. 3.1). These data suggest that EGF regulation of TIF2 coactivation is not at the transcriptional level. Our published data indicate that EGF signaling through MAP kinase increases TIF2 coactivation of AR transactivation in recurrent prostate cancer (79).

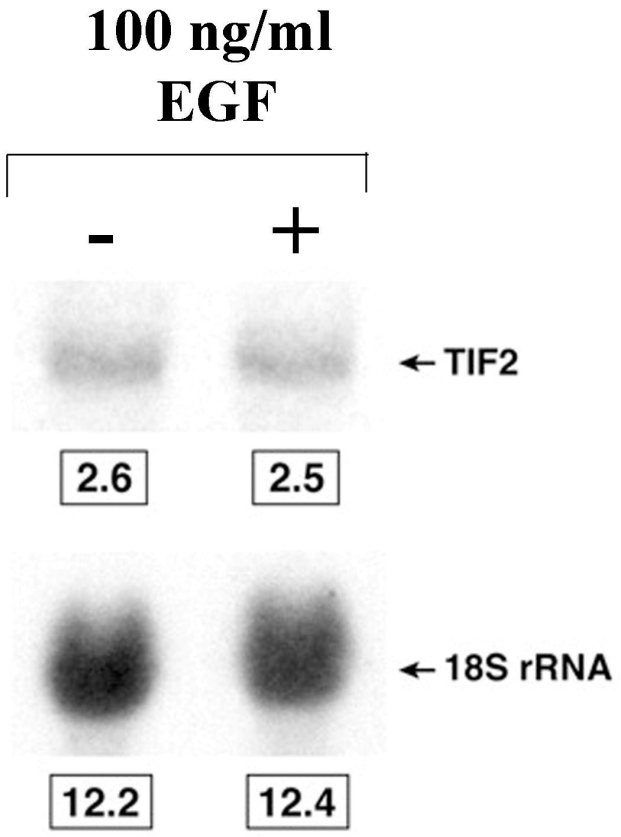

Figure 3.1. Analysis of EGF-mediated TIF2 mRNA regulation. CWR-R1 cells were incubated without and with EGF (100 ng/ml) in the absence of serum for 24 h. 15 µg of RNA was fractionated on 1% agarose gels, transferred to a nylon membrane, and hybridized with 32P-labeled TIF2 and 18S ribosomal RNA cDNAs. Densitometry values of the specific bands are included below the lanes.

TIF2- MEDIATED AR TRANSCRIPTIONAL ACTIVATION

To determine whether TIF2/GRIP1 is required for AR transactivation induced by DHT and EGF in CWR-R1 cells, we used RNA interference to inhibit endogenous TIF2/GRIP1 expression. An siRNA mixture consisting of 12-30 base pairs double-stranded RNA coding for TIF2/GRIP1 was transiently transfected into CWR-R1 cells together with pCMVhAR and PSA-Enh-Luc. TIF2 siRNA directed at nucleotides 197-376 inhibited AR transactivation of PSA-Enh-Luc by 5-fold, whereas a glyceraldehyde-3-phosphate dehydrogenase siRNA mixture had no effect (Fig. 3.2). Specificity for the TIF2 siRNA inhibition was established by cotransfecting pSG5-TIF2 with 10 nM TIF2 siRNA. Partial recovery of AR transactivation of PSA-Enh-Luc activity in the presence of overexpressed TIF2 provided evidence that inhibition by TIF2 siRNA was specific.

The data suggests that, in conditions of androgen deprivation, there is a direct link between AR transcriptional activity, increased p160 coactivator levels and EGF signaling in recurrent prostate cancer.

DOMINANT NEGATIVE INHIBITION OF AR TRANSACTIVATION

To study the role of AR in CWR-R1 cells, a dominant negative approach was used. ARΔ142-337 contains a deletion in the NH_2-terminal region that results in loss of AR transactivational activity and inhibits wild-type AR possibly due to dimerization and loss of binding of one or more coactivators. Transient transfection of CWR-R1 cells with ARΔ142-337 decreased DHT and DHT plus EGF-mediated MMTV-Luc transcription, MMTV contains an AR target promoter and it is transactivated by endogenous AR.

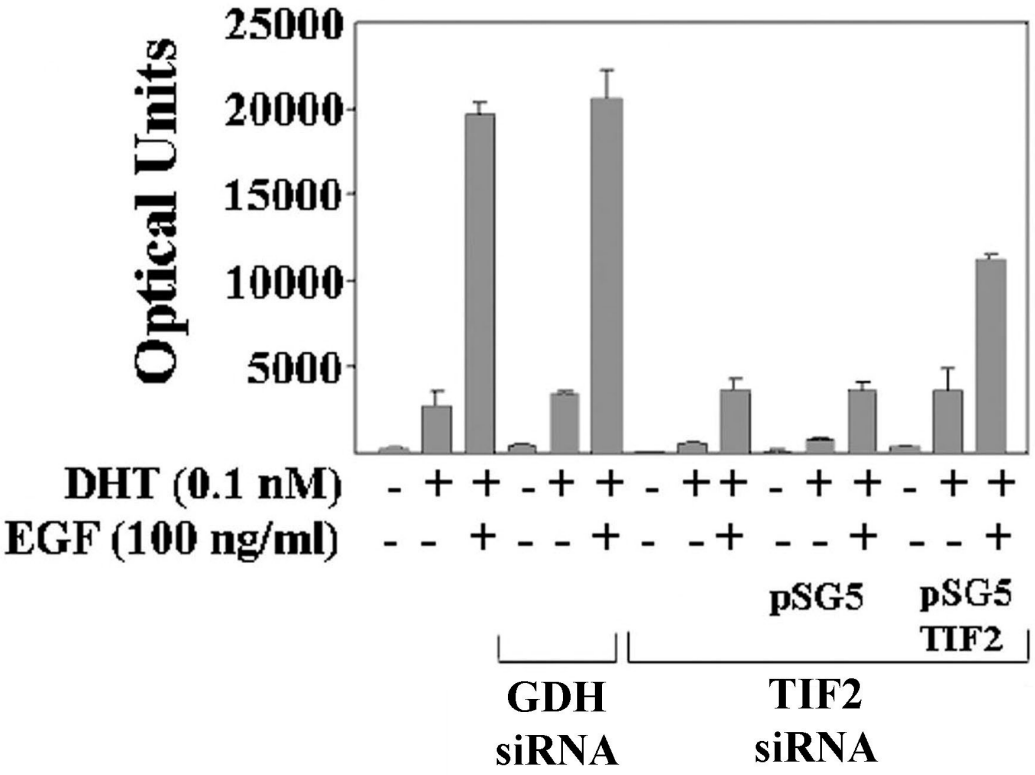

Figure 3.2. Interference of AR transcriptional activity by Tif2 siRNA. CWR-R1 cells were transfected with 0.1 μg PSA-Luc reporter and pCMVhAR with and without 10 nM GAPDH siRNA or TIF2 siRNA in the absence and presence of 0.05 μg of pSG5 or pSG5-TIF2 using Effectene as described in the Experimental Procedures section. Cells were incubated for 24 h with and without 0.1 nM DHT in the absence and presence of 100 ng/ml EGF. The data are representative of four independent experiments.

CWR-R1 cells (Fig. 3.3). Suppression of AR using ARΔ142-337 delivered via lentiviral vectors was used to test the hypothesis that prostate cancer recurrence can be delayed or prevented by interfering with AR function. Our unpublished studies show that lentiviral vectors can achieve high level expression of ARΔ137-142, decrease plasmid luciferase expression from the MMTV promoter, and decrease CWR-R1 cell proliferation in culture. Studies are underway to determine the role of AR in CWR-R1 tumor formation *in vivo*.

HUMAN KALLIKREIN 2 (HK2) AS A MARKER OF ANDROGEN REGULATED GENE TRANSCRIPTION

Insulin-like growth factor binding protein-5 (IGFBP5), homeobox gene Nkx 3.1, AR coactivator ARA-70, human kallikrein 2 (hK2) and cell cycle genes Cdk1 and Cdk2 are androgen regulated in the CWR22 human prostate cancer xenograft (50). The PSA-Enh-Luc reporter gene is activated only weakly in CWR-R1 cells. We explored genes containing AREs to determine a marker of endogenous AR activity in CWR-R1 cells. In light of the fact that hK2 is useful in clinical diagnosis and prognosis of prostate cancer (152) and that an ARE in the 5′ far upstream promoter region of the hK2 gene was identified as crucial for its regulation in LNCaP cells (153), we measured hK2 mRNA levels in the absence and presence of DHT.

To demonstrate that ARΔ142-337 inhibits endogenous AR in the CWR1 recurrent prostate cancer cell line, we performed gene profile studies of these candidate genes. Northern blots suggested that hK2 levels increased ~2 fold in response to androgen in CWR-R1 cells. To confirm this, LacZ-transduced CWR-R1 cells were

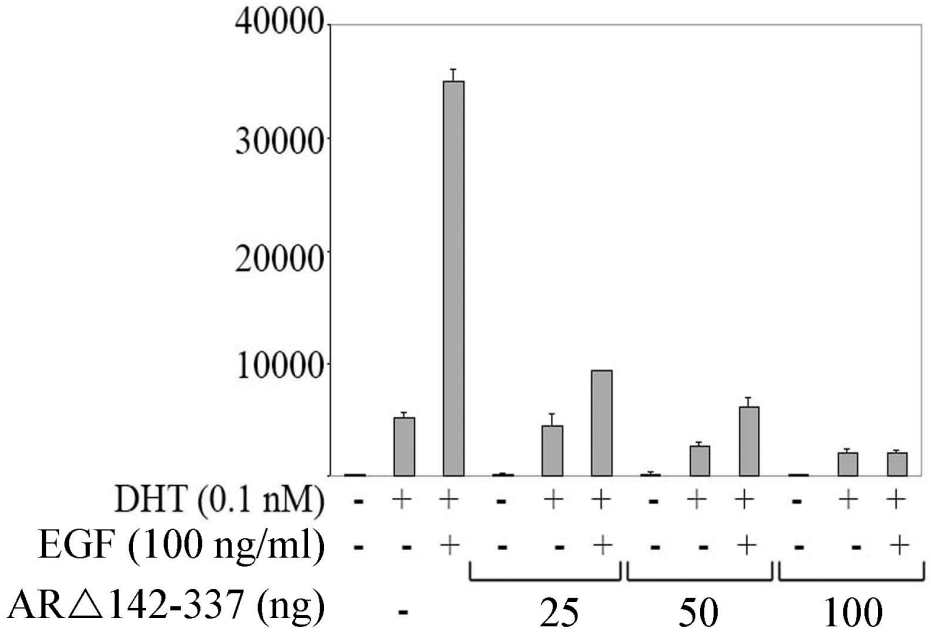

Figure 3.3. Inhibition of endogenous AR activity by the dominant negative ARΔ142-337. CWR-R1 cells were transfected with 0.1 μg MMTV-Luc reporter in the presence or absence of ARΔ142-337 as indicated using Effectene. Cells were incubated for 24 h with and without 0.1 nM DHT in the absence and presence of 100 ng/ml EGF and luciferase activity determined. The data are representative of four independent experiments.

seeded, serum-starved for 24h and treated with or without 0.1nM DHT and harvested for one-step real time-RT PCR analysis. Results indicate 2-fold induction of hK2 mRNA (Fig. 3.4) in response to DHT suggesting hK2 transcription is a marker of AR transcriptional activity in the recurrent CWR-R1 cancer cell line.

Our data indicate that in CWR-R1 cells hK2 may be a suitable gene for studies involving endogenous AR activity in castration-recurrent cell lines. Improving the accuracy of testing for prostate cancer by combining the conventional PSA test with PSA derivatives or with other markers, such as hK2, could improve the current PSA test to accurately estimate cancer volume and preoperative staging.

siRNA-Mediated Inhibition of AR expression and transactivation

As an alternative approach to inhibiting endogenous AR, siRNA targeting AR was used to determine the requirement for AR transcriptional activation. Four siRNA duplexes targeting AR mRNA sequence were transfected into CWR-R1 cells to inhibit AR-mediated transcriptional activation of the MMTV-Luc reporter gene. As expected, DHT increased AR transcriptional activity ~100-fold, with a further 3-fold increase after addition of EGF (Fig. 3.5). A similar effect of DHT with and without EGF on AR transcriptional activity was seen after transfection of a control siRNA oligonucleotide that did not reduce AR levels (Fig. 3.5A and 3.5B, lane 5). However, AR targeted siRNA oligonucleotide-3 that reduced AR levels (Figure 3.5B, lane 3) greatly reduced AR transactivation, with only a 2-fold increase in activity remaining in the presence of DHT and a ~12 fold increase in response to DHT and EGF (Fig. 3.5A).

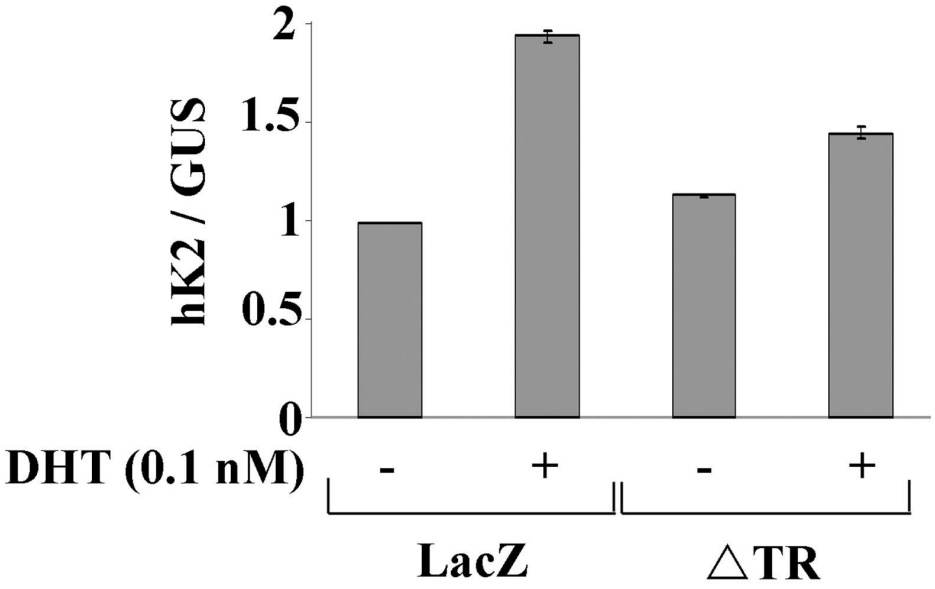

Figure 3.4. Androgen regulation of hK2 mRNA is CWR-R1 cells. LacZ or Δ142-337-transduced CWR-R1 cells were serum starved for 24 h and treated with and without 0.1 nM DHT for 24 h. Shown are relative levels hK2 mRNA determined from total RNA by real-time PCR as described in Experimental Procedures from duplicates of two 6 cm dish cultures extrapolated from standard curves and threshold cycle Ct values and expressed as ratios of target gene to control gene β-glucuronidase (GUSB).

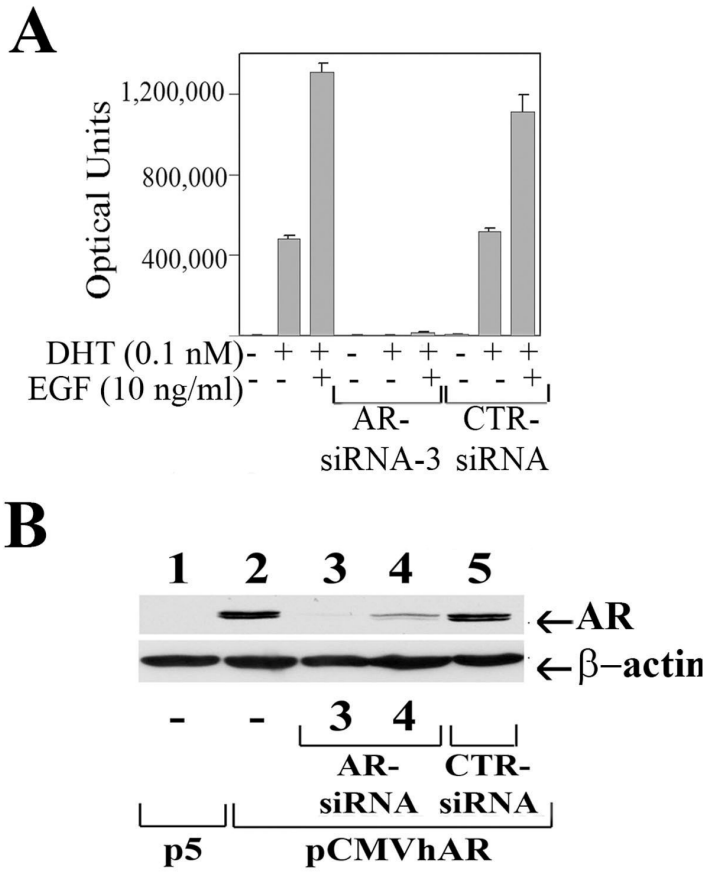

Figure 3.5. Inhibition of DHT and/or EGF stimulated AR transcriptional activity using AR siRNA. (**A**) CWR-R1 cells were transfected with 0.1 μg MMTV-Luc with or without 10 nM AR siRNA-3 or control siRNA (CTR). The next day cells were cultured for 24 h with or without 0.1 nM DHT plus 10 ng/ml EGF and luciferase activity was determined. In (**B**), COS cell extracts (30 μg protein/lane) from cells transfected with pCMV5 empty vector (p5, lane 1), or pCMV-AR in the absence (lane 2) or presence of 10 nM AR-siRNA-3 (lane 3), AR-siRNA-4 (lane 4) or control siRNA (lane 5), were analyzed by immunoblot using AR52 (2.5 μg/ml) and β-actin antibodies (1:5000 dilution).

Duplex 3 targeted an RNA sequence complementary to the hinge region of the AR and was used for subsequent cloning in viral vectors.

GENERATION AND CHARACTERIZATION OF AR-SIRNA-SCAAV AND CONTROL-SIRNA-SCAAV

A scAAV type II was generated to target endogenous AR protein in CWR-R1 cells as indicated in the "Experimental Procedures" section. Slot blot analysis was performed to confirm viral titer (Figure 3.6A). To evaluate transduction efficiency of the virus, flow cytometry was performed on infected CWR-R1 cells. Viral particles (10^3 and 10^4) were used as the initial titer per cell. A green fluorescent protein (GFP) containing scAAV serotype II (p-hpa-trs-SKAAV2) was used for flow cytometry analysis. One day after infection ~72% of the cells were GFP positive at a viral dose of 10^4 and ~83% GFP positive using 10^3 viral particles/cell.

To characterize additional infectivity properties of the virus in CWR-R1 cells, cells were plated in L-Polylysine-coated plates. Transduction efficiency was significantly reduced as only ~28% of the cells were GFP positive at 10^3 virus particles/cell 24 h after infection.To evaluate the timing of transduction, cells were left in culture after infection for 9 days and flow cytometry was performed. At 10^3 virus particles/cell the GFP positive cell population decreased about ~62% from initial infection demonstrating that, due to the episomal mechanism of viral transduction, transgene expression was lost in the cultured cells. To explore optimal methods for scAAV transduction, experiments were performed by infecting cells without inoculation of the virus-containing media. Flow cytometry showed ~80% transduction efficiency.

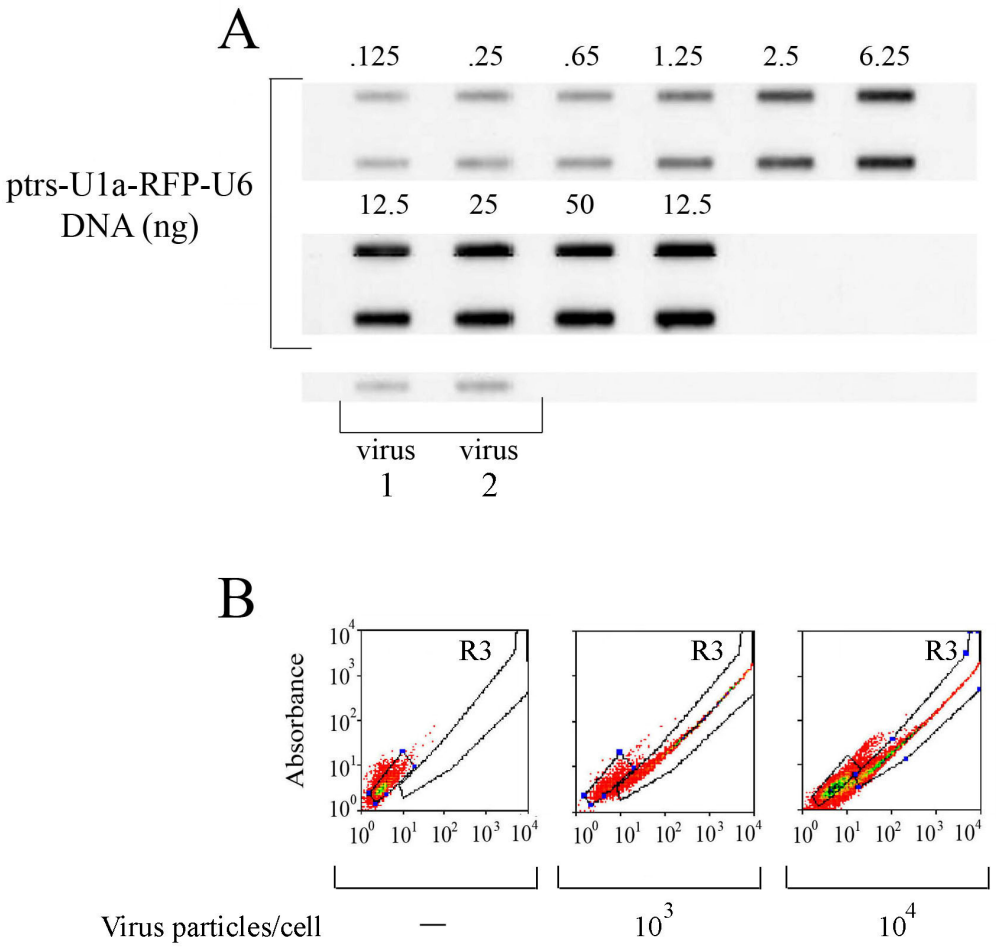

Figure 3.6. Slot-blot analysis of AR and control scAAV-siRNA viral titers. **(A)** Increasing amounts of vector DNA were transferred using a slot-blot apparatus to a nitrocellulose membrane. DNA was crosslinked and the blot probed with radioactively labeled linearized ptrs-U1a-RFP-U6 for 2 h. **(B)** Transduction efficiency of a GFP-scAAV (serotype 2) virus. CWR-R1 cells were seeded and left uninfected (left panel) or infected with 10^3 (middle panel) or 10^4 (right panel) virus particles/cell of p-hpa-trs-GFP-SKAAV2. Cells were collected the next day for flow cytometry analysis of GFP expression. Cells gated in cannel R3 are GFP positive.

infection (Fig. 3.6B, third panel). Western blots were performed 5 days after infection in CWR-R1 cells (Fig. 3.7). The data indicate transduction of scAAV-siRNA under these conditions results in partial inhibition of AR expression 2 days after infection of CWR-R1 cells.

scAAV uptake and transduction varies among cell types. To test whether the scAAV-ARsiRNA inhibited endogenous AR expression in prostate cancer cell lines LNCaP, LNCaP-C42 and LAPC4 cells were infected with the scAAV-CTR or scAAV-ARsiRNA. 48 h after infection, AR protein expression was decreased in the presence and absence of DHT (Fig. 3.8 A-C). These data validate scAAV as a mechanism for siRNA delivery and AR protein inhibition in several prostate cancer cell lines. This tool can be used in investigating the role of AR in prostate cancer progression and recurrence *in vitro*.

AR-DEPENDENT CWR-R1 PROSTATE CANCER CELL PROLIFERATION

We investigated the requirement for AR in castration-recurrent prostate cancer cell growth by performing proliferation studies using CWR-R1 cells infected with control or AR targeted siRNA-scAAV in the absence and presence of DHT and/or EGF. CWR-R1 cells were infected and viral containing media inoculated prior to incubation form 30 min at room temperature. AR-siRNA-scAAV infected cells grew more slowly than control cells in the absence and presence of 0.1 nM DHT (Figure 3.9A). The >90% reduction in AR levels in CWR-R1 cells treated without (Figure 3.9B, upper panel) and with DHT (Figure 3.9B, lower panel) assayed by immunoblot 2 and 5 days after AR-siRNA-scAAV infection suggested that CWR-R1 cell growth was stimulated by AR both in the absence and presence of androgen.

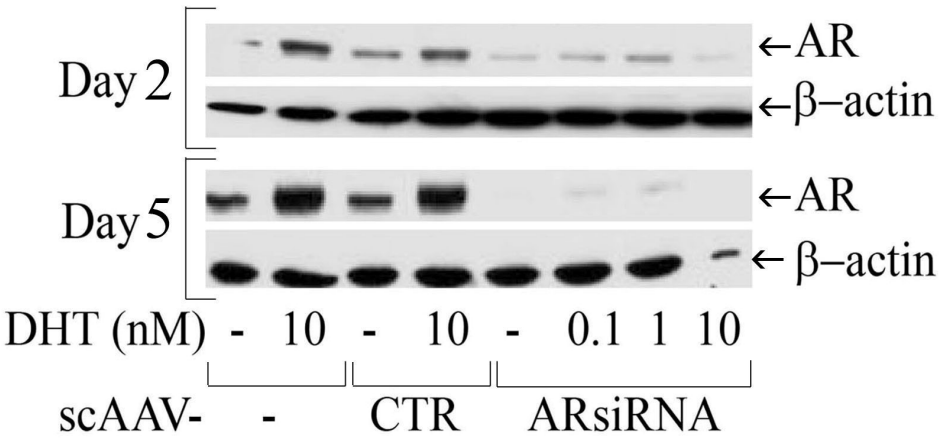

Figure 3.7. Inhibition of AR expression by AR-siRNA-scAAV in CWR-R1 cells. $1x10^6$ cells/6 cm dish were infected 24 h after plating with AR- or CTR-siRNA-scAAV at 10^3 virus particles/cell as described in "Experimental Procedures" with slight modifications. After infection, cells were incubated for 24 h and treated with or without 0.1, 1 or 10 nM DHT as indicated. An additional group was mock-infected and treated with or without 10 nM DHT. Immunoblots of endogenous AR in CWR-R1 cells were performed 2 and 5 days after infection and β-actin was used as a loading control.

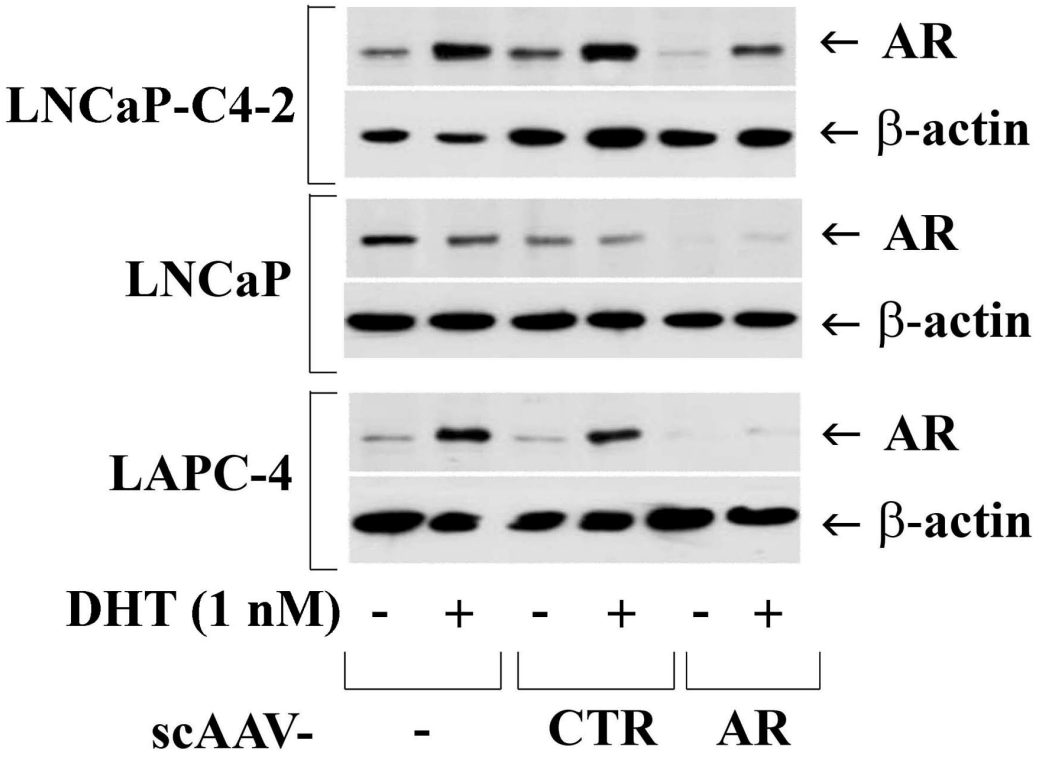

Figure 3.8. Inhibition of AR expression by AR-siRNA-scAAV in LNCaP-C4-2, LNCaP, and LAPC-4 cells. LNCaP-C4-2, LNCaP or LAPC-4 prostate cancer cells in $1x10^6$ cells/6cm dishes were infected with AR- or CTR-siRNA-scAAV at 10^3 virus particles/cell as described in "Experimental Procedures". The next day cells were treated with and without 1 nM DHT as indicated. An additional group was mock-infected and treated with and without 1 nM DHT. Immunoblots of endogenous AR were performed 2 and 5 days after infection and β-actin was used as a loading control.

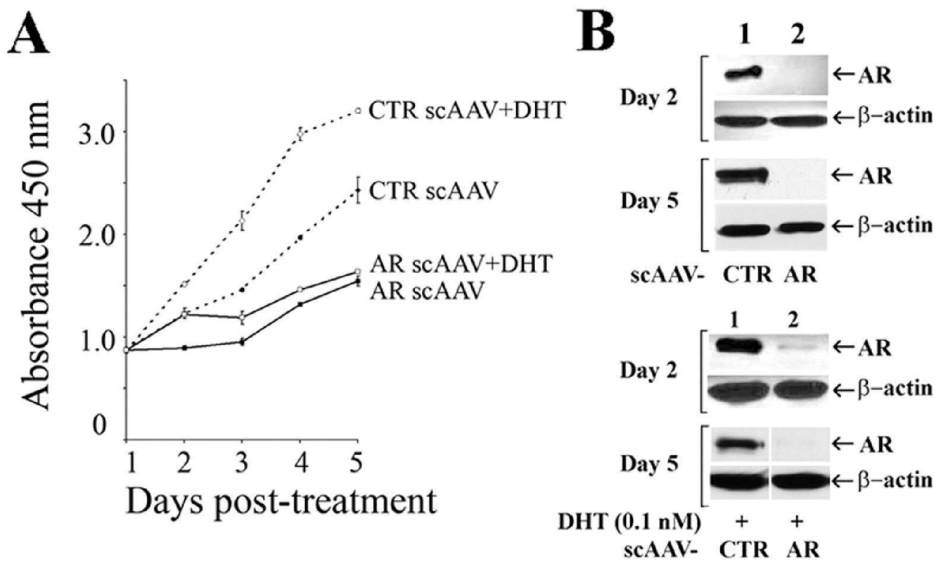

Figure 3.9. Inhibition of CWR-R1 cell growth by AR-siRNA-scAAV in the absence and presence of DHT. **(A)** CWR-R1 cells were infected with control or AR targeted siRNA-scAAV (10^3 virus particles/cell) and cultured with or without 0.1 nM DHT. Colorimetric assays at 450 nm using WST-8 reagent were performed in duplicate to measure cell proliferation daily up to 5 days. Medium was replaced every other day with or without 0.1 nM DHT as indicated. **(B)** Immunoblots of endogenous AR in CWR-R1 cells were performed 2 and 5 days after infecting cells with control siRNA scAAV (CTR) or AR-siRNA-scAAV in the absence (top panels) and presence of 0.1 nM DHT (bottom panels).

EGF also stimulated control CWR-R1 cells to grow faster than cells infected with AR-siRNA-scAAV (Figure 3.10A, left panel). The reduction in AR levels determined by immunoblot of EGF treated CWR-R1 cells 2 and 5 days after AR-siRNA-scAAV infection (Figure 3.10A, right panel) provided evidence that EGF stimulation of CWR-R1 cell growth was mediated in part by AR. However, the attenuated but significant growth response of AR-siRNA-scAAV infected cells to EGF suggested that EGF also stimulated cell proliferation through signaling mechanisms that are independent of AR. The stimulatory effect of EGF together with DHT on growth of control and AR-siRNA-scAAV infected cells was greater than either hormone alone and approached maximal levels within 3 days (Figure 3.10B, left panel). Growth of AR-siRNA-scAAV infected cells treated with DHT and EGF was less attenuated compared to control cells, even though AR levels were reduced as shown by immunoblot (Figure 3.10B, right panel). The results indicate that AR increases CWR-R1 prostate cancer cell growth in response to DHT or EGF, and that EGF and DHT act synergistically through AR.

ANDROGEN-INDEPENDENT AR TRANSACTIVATION AND EGF-MEDIATED INCREASE IN FLAG-(AR507-660) EXPRESSION

To pursue evidence that AR functions in castration-recurrent prostate cancer in the absence of androgen, we tested whether EGF can increase endogenous AR transcriptional activity in CWR-R1 cells using an MMTV-Luc reporter vector in the absence of DHT. In response to EGF alone, the ~3 fold increase in AR transcriptional activity in the presence of control siRNA was abrogated by AR duplex siRNA oligonucleotide-3 (Figure 3.11). The results indicate that EGF can activate AR in the

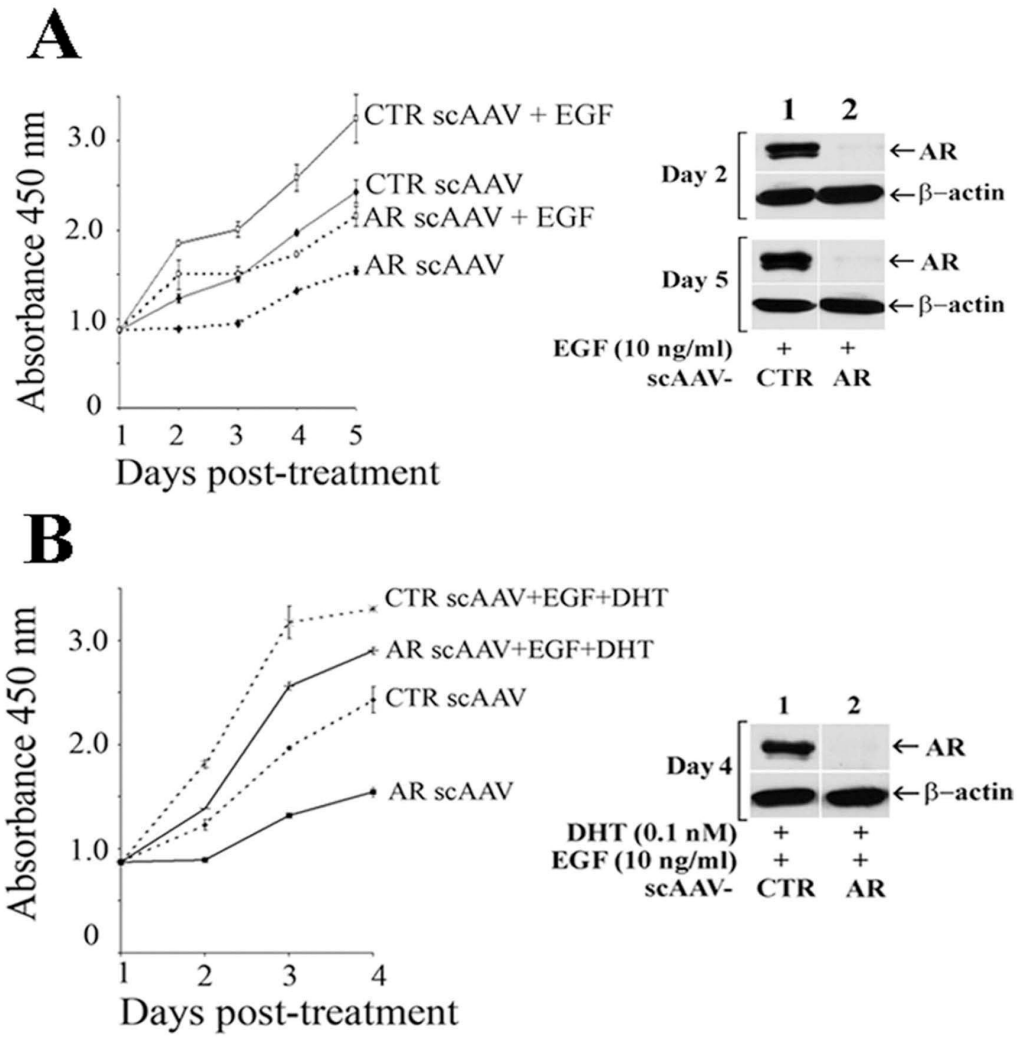

Figure 3.10. Inhibition of EGF and EGF plus DHT stimulated CWR-R1 cell growth using AR-siRNA-scAAV. (**A**) CWR-R1 cells were infected with control or AR targeted siRNA-scAAV and cultured with or without 10 ng/ml EGF. Cell proliferation was assayed in the absence or presence of 10 ng/ml EGF as described in Fig. 1 (left panel). AR expression was determined by immunoblotting CWR-R1 cell lysates 2 and 5 days after infecting cells with control siRNA-scAAV or AR-siRNA-scAAV in the presence of EGF (right panels). (**B**) CWR-R1 cells were infected with control or AR targeted siRNA-scAAV and treated with and without 10 ng/ml EGF and 0.1 nM DHT (left panel). Immunoblots of CWR-R1 cell AR were performed 4 days after infecting cells with control siRNA-scAAV or AR-siRNA-scAAV in the presence of 10 ng/ml EGF and 0.1 nM DHT (right panel).

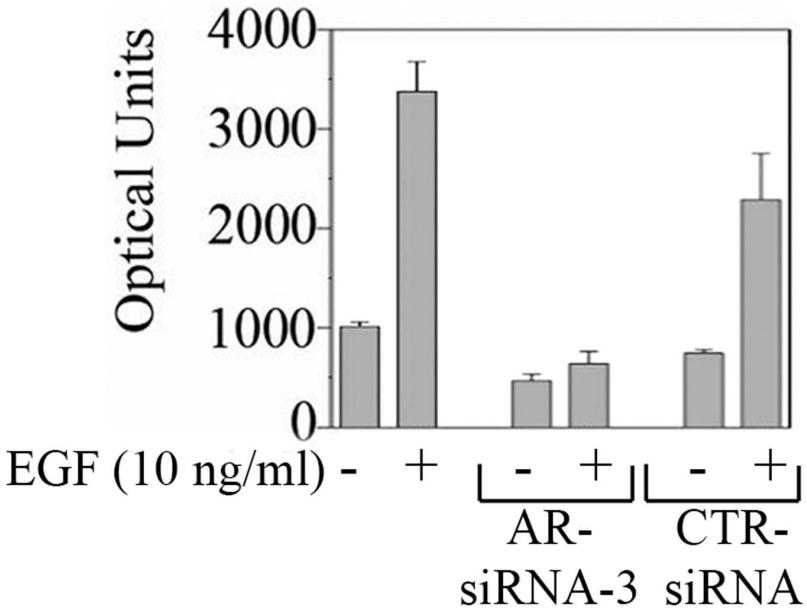

Figure 3.11. Inhibition of EGF stimulated AR transcriptional activity using AR siRNA. CWR-R1 cells were transfected with 0.1 μg MMTV-Luc with and without 10 nM AR siRNA-3 or control siRNA (CTR). The next day cells were cultured for 24 h with and without 10 ng/ml EGF and luciferase activity was determined.

CWR-R1 prostate cancer cell line in the absence of androgen and that EGF and DHT act synergistically to increase AR transcriptional activity. These data, together with the cell growth studies presented above, support the hypothesis that AR activation by EGF is sufficient to drive prostate cancer cell growth.

To determine whether EGF directly affects AR expression, western blots were performed in COS cells expressing pCMV-AR507-660. An EGF-dependent increase of pCMV-AR507-660 was evidenced up to 10 h after the onset of treatment (Figure 3.12, left panel). To verify that the effects were not due to EGF effects on the CMV promoter, pCMV-Flag-CHIP was transfected into COS cells and treated with EGF. The similar levels of pCMV-Flag-CHIP suggest that EGF effects were independent of the CMV promoter. These data indicate that there are in a time dependent effects of EGF on AR and post-transcriptional modifications that may play a role on AR transcriptional activity.

REAL-TIME RT-PCR ANALYSIS OF ANDROGEN-MEDIATED REGULATION OF EGF TRANSCRIPTION

Prostate cancer cell lines have been shown to synthesize and secrete EGF and related peptides (154). Recurrent CWR22 tumors express high levels of EGF-related ligands compared with the androgen-dependent (98), and increased immunostaining of TGF-α was found in recurrent CWR22 xenografts (155). To determine whether EGF was androgen-regulated in CWR-R1 cells, real-time PCR analysis was performed in the presence and absence of 0.1 nM DHT. Standard curves were generated for EGF and human GUSB (beta-glucuronidase) genes using fluorescent probes in a one-step reverse transcription real-time PCR reaction. Efficiency coefficients for the two genes were

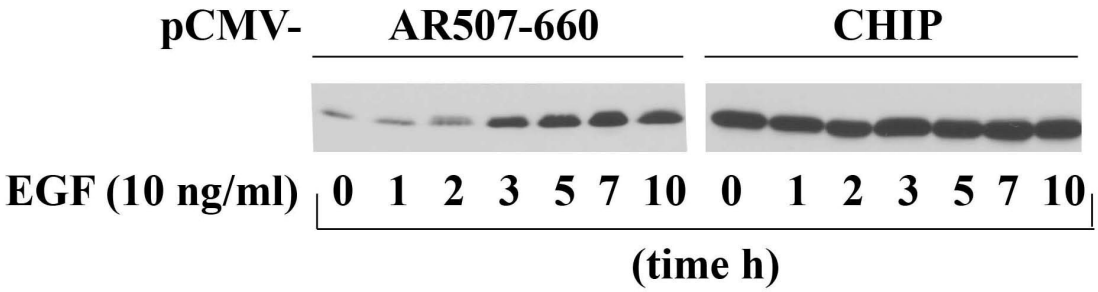

Figure 3.12. Increase in pCMV-AR597-660 by EGF. COS cells ($2.5x10^6$/10 cm dish) were transfected with 2 µg pCMV-AR-(507-660) or pCMV-CHIP. Cells were serum depleted for 24 h, treated with and without 10 ng/ml EGF for 1, 2, 3, 5, 7 and 10 h, collected and lysed in the presence of phosphatase inhibitors for immunoblotting. AR52 antibody was used to detect pCMV-AR507-660 and a CHIP specific antibody to detect pCMV-CHIP.

calculated and relative quantitation was performed to compare the normalized cross point values for EGF. We found that EGF expression in CWR-R1 cells (Figure 3.13) was not androgen regulated. This suggests that EGF mediated effects in CWR-R1 cells are not due to increased EGF transcription under conditions of androgen deprivation.

EGF DEPENDENT PHOSPHORYLATION WITHIN THE AR NH_2-TERMINAL AND DNA BINDING DOMAIN (DBD) FRAGMENT AR-(507-660)

Sequence analysis using NetPhos 2.0 (27) indicated 15 consensus serine, threonine or tyrosine phosphorylation sites between AR residues 507-660 that comprise part of the AR NH_2-terminal region, the DNA binding domain and hinge region (Figure 3.14 A). Immunoblots of wild-type Flag-AR-(507-660) expressed in COS cells revealed a 21 kDa protein, and after treatment with EGF, an additional slower migrating 23 kDa form (Figure 3.14B, lanes 1–3). The EGF dependent slower migrating form was eliminated by treatment with λ-phosphatase in the absence, but not in the presence of phosphatase inhibitors (Figure 3.14 B, lanes 3–8). The slower migrating 23 kDa band was also observed in response to EGF with Flag-AR-(507-660)-C576A, which has a cysteine mutation in the first zinc module that eliminates DNA binding (see data in Figure 3.21, lanes 3–4). The appearance of an EGF-dependent and phosphatase-sensitive slower migrating form of Flag-AR-(507-660) indicated that EGF induces phosphorylation at one or more sites between AR residues 507-660 independent of AR binding to DNA.

To identify the EGF-dependent AR phosphorylation site(s), single serine or threonine to alanine and tyrosine to phenylalanine mutations were introduced into Flag-AR-(507-660) at the consensus phosphorylation sites highlighted in Figure 3.14A.

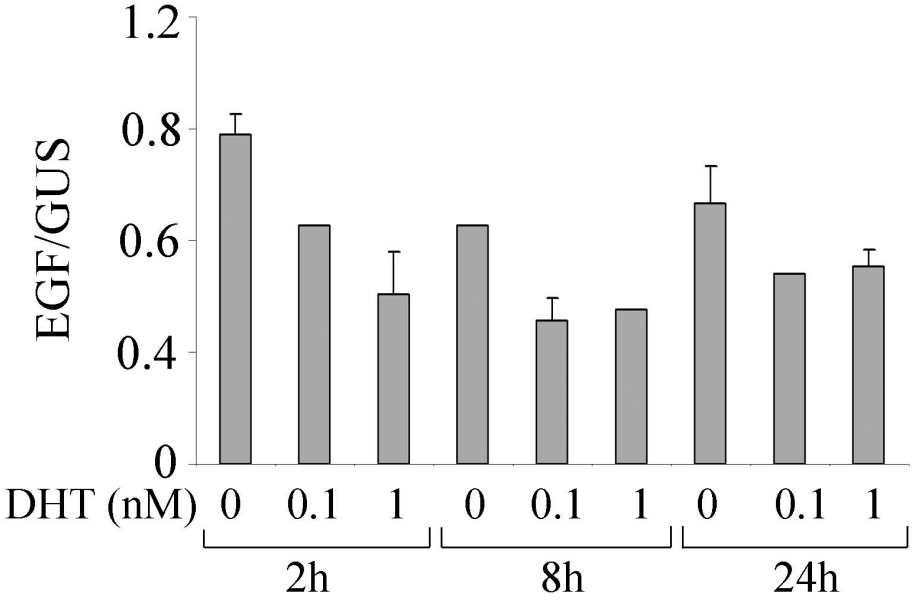

Figure 3.13. EGF mRNA levels in CWR-R1 cells with and without DHT. CWR-R1 cells were serum starved for 24 h and treated with and without 0.1, 1 or 10 nM DHT for 2, 8 or 24 h. Shown are relative levels EGF mRNA determined from total RNA by real-time PCR as described in the section "Materials and Methods" from duplicates of two 6 cm dish cultures extrapolated from standard curves and threshold cycle Ct values and expressed as ratios of target gene to control gene β-glucuronidase (GusB).

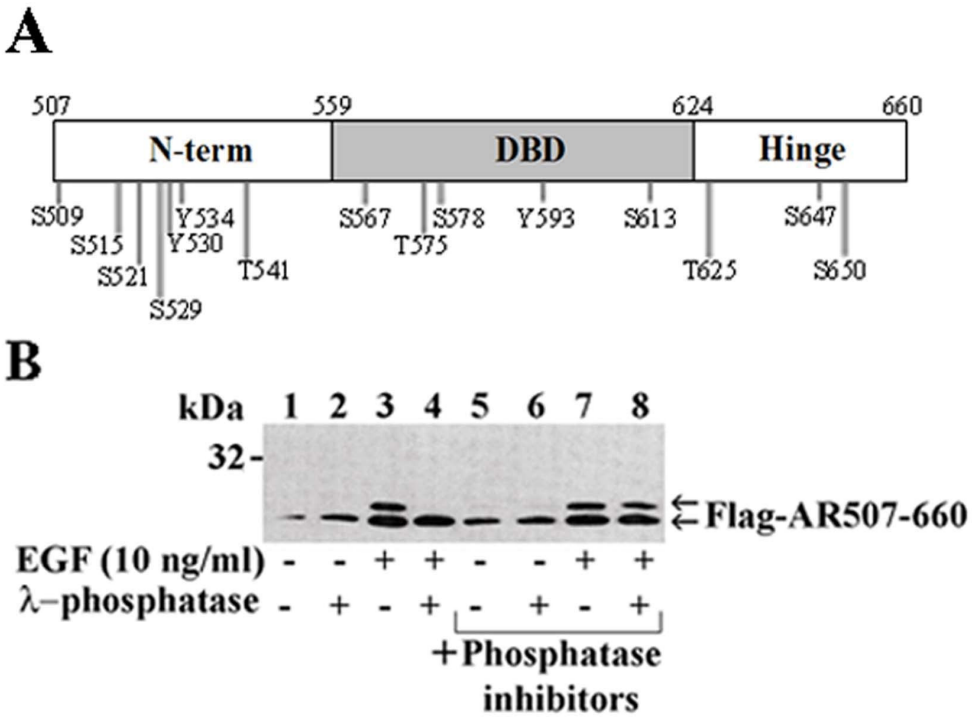

Figure 3.14. EGF-dependent phosphorylation of AR507-660. (**A**) Schematic representation of 15 predicted phosphorylation sites in part of the AR NH_2-terminal region (N-term), DNA binding domain (DBD) and hinge region that were mutated in Flag-AR-(507-660) and tested for band shift on immunoblots. (**B**) COS cells were transfected with 2 μg Flag-AR-(507-660), serum-depleted for 24 h and treated for 5 h with and without 10 ng/ml EGF as indicated. Cells were harvested and lysates incubated with and without 2.5 units λ-phospatase for 30 min at 30ºC in the absence (lanes 1-4) and presence of phosphatase inhibitors (lanes 5–8).

Immunoblots of cell extracts before and after treatment with EGF indicated that only the AR NH_2-terminal MAP kinase consensus S515A mutation eliminated the slower migrating 23 kDa form of Flag-AR-(507-660) when assayed in COS and CWR-R1 cells (Figure 3.15A, lanes 1–4). This result provided evidence that AR is phosphorylated at Ser-515 in response to EGF. In addition, the PKC consensus site mutation S578A in the AR DNA binding domain increased the relative proportion of the slower migrating 23kDa phospho-Ser-515 form in the presence of EGF (Figure 3.15 A, lanes 5-6). MAP kinase dependent phosphorylation at AR Ser-515 was supported by the decrease in intensity of the slower migrating 23 kDa band after treatment with both EGF and increasing concentrations of the MAP kinase inhibitor, U0126 (Figure 3.15 B).

AR phosphorylation at Ser-578 was indicated by the reactivity of an AR phospho-Ser-578 specific antibody that recognized two AR-(572-586) phospho-Ser-578 conjugated peptides used as immunogens but not unphosphorylated AR-(572-586) (Figure 3.16A, lanes 3-5). The AR phospho-Ser-578 specific antibody also recognized the faster migrating 21 kDa form of wild-type AR-(507-919) but not the S578A mutant (Figure 3.16B, upper panel, lanes 2-5), whereas both forms were detected using the AR52 antibody (Fig. 3.16B, lower panel). Treatment with λ-phosphatase reduced the reactivity of the phospho-Ser-578 antibody reactivity with wild-type AR-(507-919) (data not shown).

These results suggest that the EGF-dependent increase in AR transcriptional activity and CWR-R1 cell growth are associated with MAP kinase dependent phosphorylation at AR Ser-515 in the NH_2-terminal region, and modulation by phosphorylation at Ser-578 in the DNA binding domain.

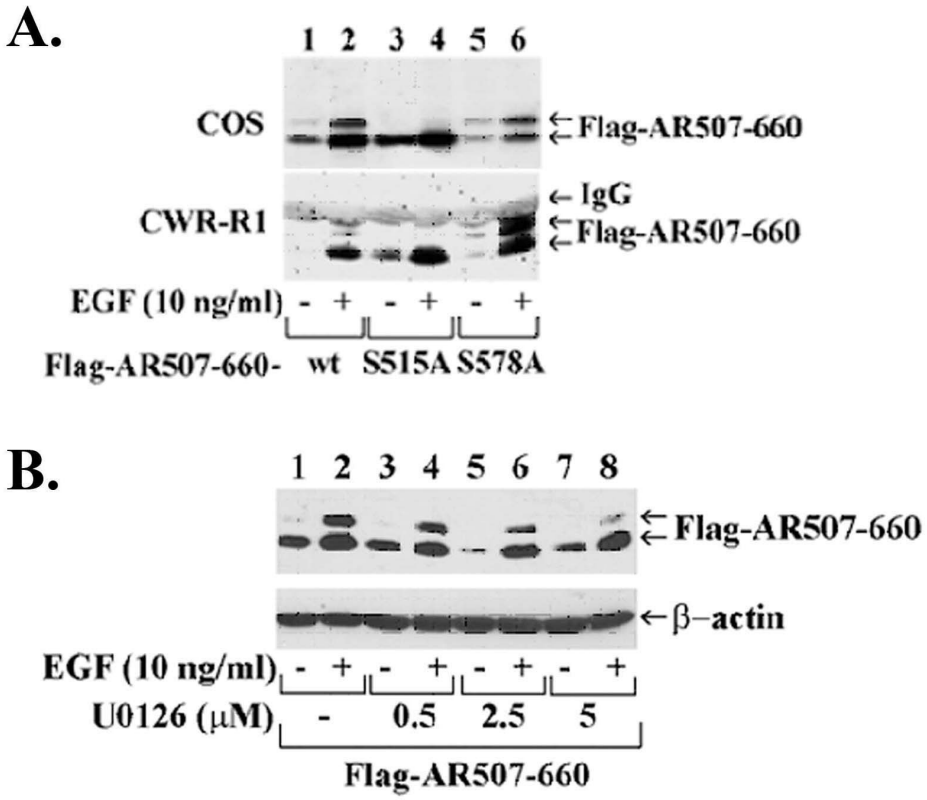

Figure 3.15. EGF dependent phosphorylation at AR Ser-515. (**A**) COS cells (upper panel) and CWR-R1 cells (lower panel) were transfected with 2 μg wild-type (wt) Flag-AR-(507-660) or the S515A and S578A mutants. Cells were serum depleted for 24 h, treated with and without 10 ng/ml EGF for 5 h, collected and lysed in the presence of phosphatase inhibitors for immunoprecipitation using Flag affinity resin. AR52 antibody was used to detect wt and mutant Flag-AR-(507-660) and an associated slower migrating band indicative of phosphorylation. Also indicated is the nonspecific IgG band. (**B**) Reduced AR Ser-515 phosphorylation by MAP kinase inhibitor, U0126. COS cells were treated in the absence (lanes 1-2) and presence of increasing concentrations of U0126 (lanes 3-8) for 1 h prior to transfection with 2 μg Flag-AR-(507-660). The next day cells were serum-starved for 24 h in the absence and presence of U0126. Cells were treated again for 5 h with and without U0126 in the absence (lanes 1, 3, 5 and 7) and presence of 10 ng/ml EGF (lanes 2, 4, 6 and 8). Flag-AR-(507-660) was detected using AR52 antibody and β-actin served as the loading control.

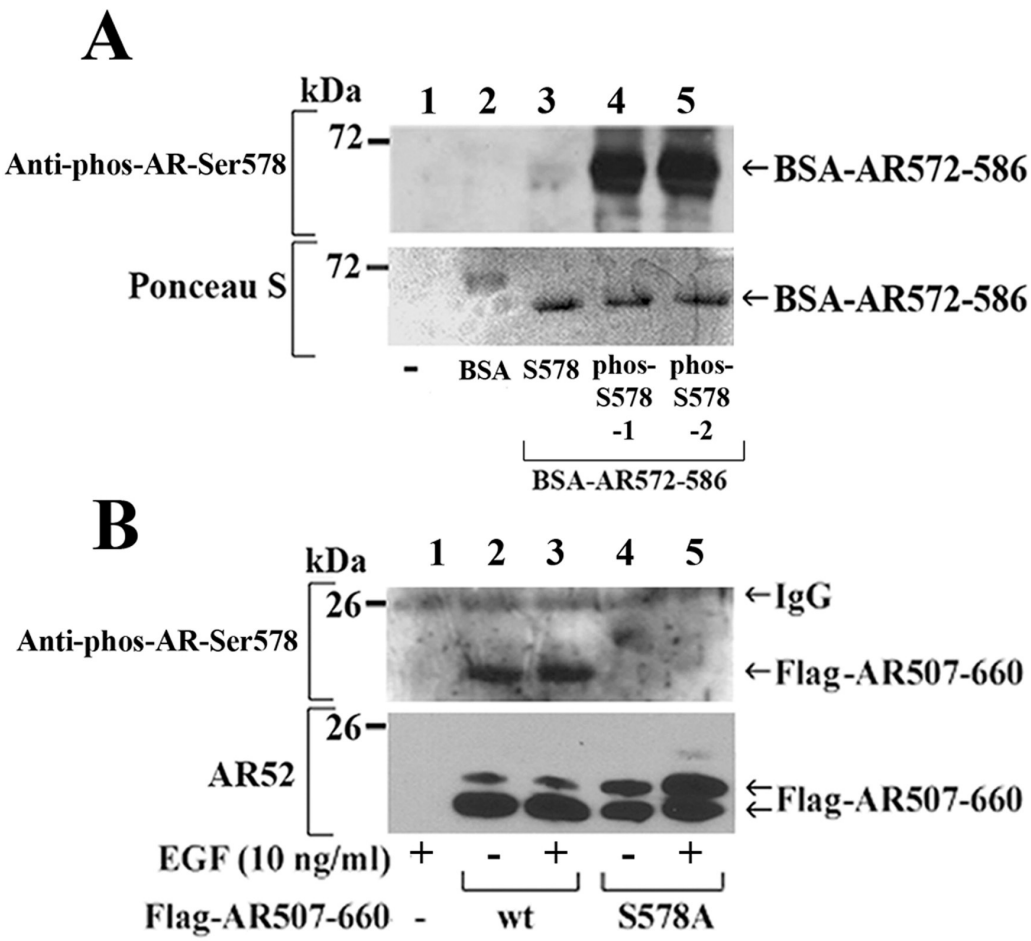

Figure 3.16. Phosphorylation at AR Ser-578. (**A**) Immunoblots without protein (lane 1), 2.5 µg BSA (lane 2), BSA-coupled nonphosphorylated AR-(572-586) peptide (lane 3) and AR-(572-586) phospho-Ser-578 peptides-1 and 2 (lanes 4 and 5) were separated on 10% acrylamide gels. Transfer blots were incubated with AR anti-phospho-Ser-578 antiserum (1:100 dilution) as described under "Experimental Procedures". Equivalent loading of the conjugated AR peptides was confirmed by staining the transfer blot with 0.2% Ponceau-S (lower panel). (**B**) COS cells were transfected with 2 µg pSG5 empty vector (-, lane 1), wild-type Flag-AR-(507-660) (lanes 2 and 3) and Flag-AR-(507-660)-S578A (lanes 4 and 5). The next day cells were transferred to serum-free medium and 24 h later treated for 5 h in the absence and presence of 10 ng/ml EGF as indicated. Cell extracts were immunoprecipitated using Flag-M2 affinity resin, separated on a 12% acrylamide gel containing SDS and transfer blots incubated with AR anti-phospho-Ser-578 antisera (1:100 dilution, upper panel) and 2.5 µg/ml AR52 antibody (lower panel) as described under "Experimental Procedures".

EFFECTS ON AR TRANSCRIPTIONAL ACTIVITY OF PHOSPHORYLATION MUTANTS IN CWR-R1 AND ISHIKAWA CELLS

We investigated further the link between EGF dependent AR phosphorylation and increased AR transcriptional activity using full-length wild-type AR and serine to alanine mutants expressed with a PSA-Enh-Luc reporter in CWR-R1 cells. AR transcriptional activity increased ~3 fold in response to EGF in the absence and presence of DHT (Figure 3.17A, upper panel). A similar response was seen with AR-S650A, which has a mutation in the previously reported Ser-650 phosphorylation site in the hinge region of AR (28, 29). AR-S515A transcriptional activity increased in response to EGF and DHT, but overall activity was less than wild-type. In contrast, EGF did not increase the transcriptional activity of AR-S578A in the absence or presence of DHT, or when the S578A mutation was combined with the S515A or S650A mutation. When assayed by immunoblot (Figure 3.17A, lower panel), expression of AR-S578A and AR-S515A was similar to wild-type AR, as was androgen dependent AR stabilization that results from the AR N/C inteaction (4, 23).

The weaker transcriptional activity of the AR-S578A DNA binding domain mutant did not result from loss of DNA binding. This was evident from AR-(1-660)-S578A, a constitutively active AR NH_2-terminal and DNA binding domain fragment that retained the transcriptional activity of wild-type AR-(1-660) using the PSA-Enh-Luc reporter (Figure 3.17B, upper panel). However, similar to results with full-length AR-S578A shown in Figure 3.17A, transcriptional activity of AR-(1-660)-S578A did not increase in response to EGF, even though expression levels of AR-(1-660)-S578A were

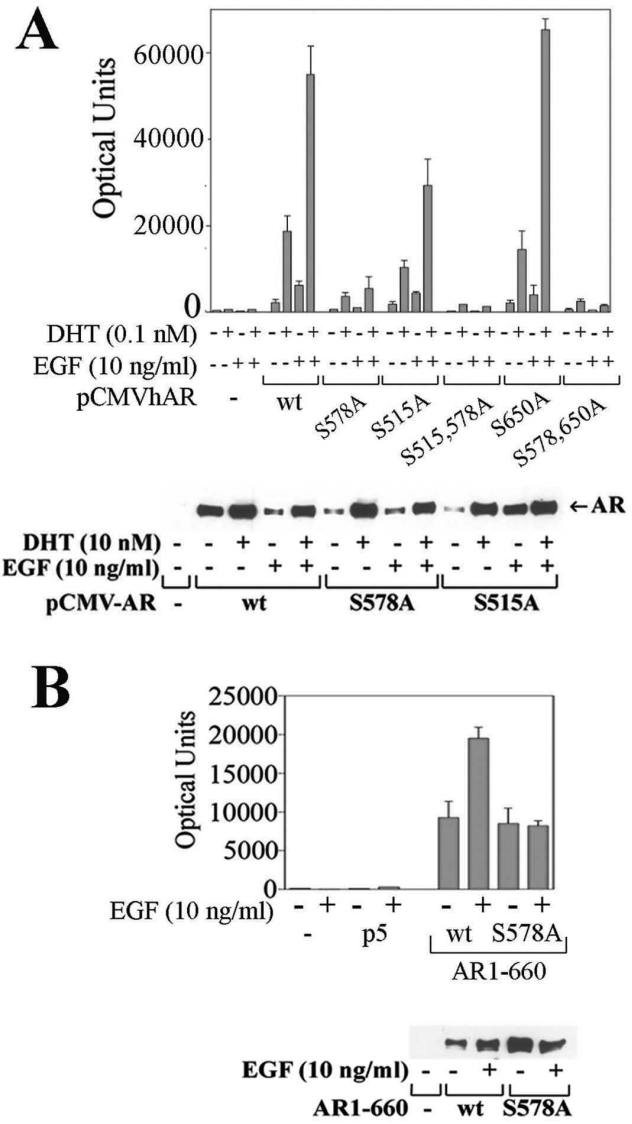

Figure 3.17 AR Ser-578 is required for EGF-induced AR transactivation in CWR-R1 cells. CWR-R1 cells were transfected with 0.1 μg PSA-Enh-Luc and **(A)** 10ng pCMV-hAR wt and S578A mutant or **(B)** 50 ng pCMV-AR-(1-660) wt and S578A mutant and incubated for 24 h with and without 10 ng/ml EGF and luciferase activity was determined (upper panel). Similar expression levels were determined by transfecting COS cells with 2 μg wt pCMV-hAR or pCMV-AR-(1-660) and the S578A mutants and incubating cells for 24 h in the absence and presence of 10 ng/ml EGF (lower panels).

similar to wild-type AR-(1-660) (Figure 3.17B, lower panel). AR-(1-660)-S578A also constitutively activated the MMTV-Luc reporter, even though full-length AR-S578A was inactive with this promoter (data not shown).

To further establish the requirement for Ser-578 in the EGF dependent increase in AR transcriptional activity, we performed transcription assays in human endometrial cancer Ishikawa cells using the PSA-Enh-Luc reporter. In the presence of increasing concentrations of DHT, AR-S578A transcriptional activity was similar to wild-type AR. This differed from CWR-R1 cells where AR-S578A transcriptional activity was less than wild-type AR. However, in agreement with results with the CWR-R1 cell line, the EGF dependent increase in wild-type AR transcriptional activity seen in the presence of DHT was diminished by the AR S578A mutation in Ishikawa cells (Figure 3.18).

The results suggest that phosphorylation at AR Ser-578 is required for the AR transcriptional response to EGF.

PKC-MEDIATED PHOSPHORYLATION WITHIN THE AR DBD AND ITS ROLE IN EGF-DEPENDENT CWR-R1 PROSTATE CANCER CELL GROWTH

Ser-578 is a predicted consensus phosphorylation site for PKC, a kinase that acts downstream of EGF signaling (30). We performed *in vitro* kinase assays using the PKC catalytic subunit with wild-type GST-AR-(1-660) and S578A mutant. PKC phosphorylation of GST-AR-(1-660) was reduced 30–35% by the S578A mutation when equivalent amounts of protein were assayed by immunoblot (Figure 3.19A, lanes 1 and 2), where Histone H1 served as a PKC substrate control (Figure 3.19A, lane 3). When averaged over multiple experiments, the S578A mutation decreased GST-AR-(1-660)

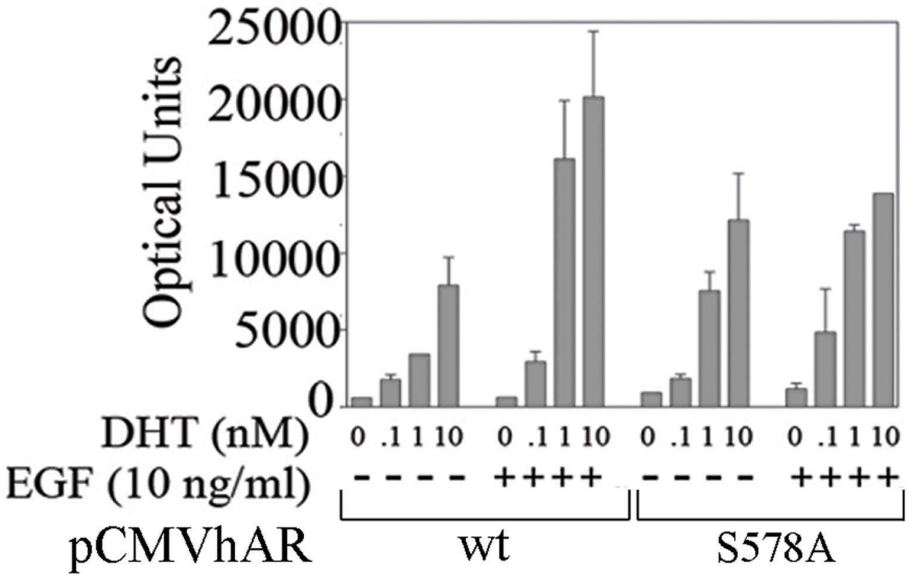

Figure 3.18. AR Ser-578 is required for EGF-induced AR transactivation in Ishikawa cells. Ishikawa cells were transfected with 0.1 µg PSA-Enh-Luc and 25 ng wild-type pCMV-AR (wt) or S578A mutant and incubated for 24 h with increasing concentrations of DHT with and without 10 ng/ml EGF as indicated and luciferase activity was determined.

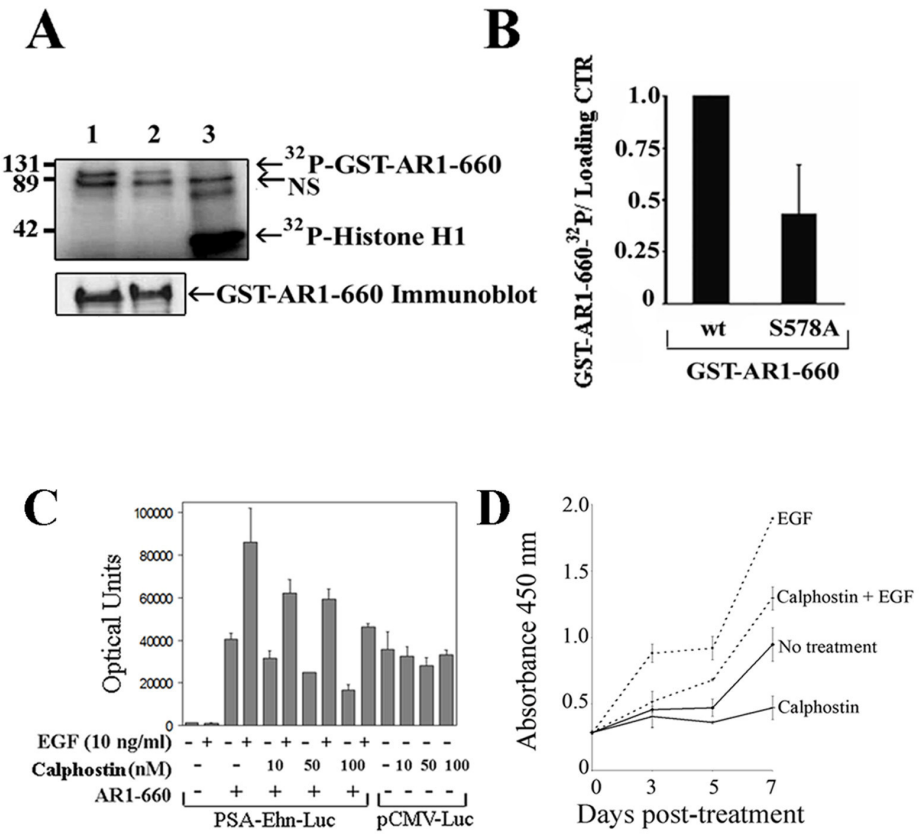

Figure 3.19. PKC-mediated phosphorylation at AR Ser-578 and EGF-dependent CWR-R1 cell growth. (**A**) In vitro kinase assays were performed using GST-AR-(1-660) (2.5 µg, lane 1) and GST-AR-(1-660)-S578A (2.5 µg, lane 2) expressed in *E. coli* and purified by adsorption to glutathione beads. Histone H1 served as a PKC substrate control (2.5 µg, lane 3). Assays were performed as described under "Experimental Procedures" using the PKC catalytic subunit in the presence of 10 µCi [γ-^{32}P] adenosine triphosphate (upper panel). Parallel immunoblots were probed with AR52 antibody (lower panel). Samples were analyzed by autoradiography and band intensities measured by densitometry. NS designates a nonspecific phosphorylated band. (**B**) Data from 4 independent experiments described in (A) were averaged. **(C)** Reduction in AR1-660 constitutive activity by PKC inhibitor Calphostin. CWR-R1 cells were treated with increasing concentrations of calphostin for 1 h prior to transfection with 25 ng pCMV-AR-(1-660) and 0.1 µg PSA-Enh-Luc, or 0.1 µg pCMV constitutive reporter. The next day cells were incubated for 24 h with and without 10 ng/ml EGF in the absence and presence of increasing concentrations of calphostin as indicated. Luciferase activity was determined 24 h later as described in Experimental Procedures. **(D)** Inhibition of CWR-R1 cell proliferation by calphostin. CWR-R1 cells were plated and serum-starved the next day for 24 h and treated as described under Experimental Procedures in serum-free media with and without 10 ng/ml EGF alone or with 50 nM calphostin, a PKC inhibitor (day 0). Media and additives were replenished every other day over 7 days. Cell proliferation indexes were measured using WST-8 reagent on days 3, 5 and 7 after seeding.

phosphorylation by ~50% (Figure 3.19B). Furthermore, transient transfection of AR1-660 in CWR-R1 cells in the presence or absence of Calphostin, a PKC inhibitor, demonstrated that PKC plays a role in the constitutive activation of the AR mutant (Figure 3.19C). There was residual EGF-induced transcriptional activation suggesting PKC-mediated effects are regulated through multiple AR sites. The EGF dependent increase in CWR-R1 cell growth (Figure 3.19D) was reduced in the presence of 50 nM Calphostin.

These results support the concept that EGF signaling through PKC phosphorylation at AR Ser-578 increases AR transcriptional activity and AR mediated CWR-R1 cell growth.

AR SER-578 PHOSPHORYLATION INFLUENCES NUCLEAR-CYTOPLASMIC SHUTTLING

The effect of Ser-578 phosphorylation on AR subcellular localization was investigated using Flag-AR-(507-660) and the S578A mutant. Immunostaining showed that wild-type Flag-AR-(507-660) was distributed between the nucleus and cytoplasm of transfected COS cells, indicative of nuclear-cytoplasmic shuttling (Figure 3.20A, left panel). The phosphomimetic Flag-AR-(507-660)-S578D was distributed similarly between the nucleus and cytoplasm (data not shown). In contrast, immunostaining of Flag-AR-(507-660)-S578A was exclusively nuclear (Figure 3.20A, right panel).

The influence of Ser-578 phosphorylation on AR compartmentalization was also investigated by comparing nuclear and cytoplasmic extracts of cells expressing Flag-AR-(507-660) and the S578A mutant before and after treatment with EGF. In agreement with the immunostaining results, wild-type Flag-AR-(507-660) was in both the nuclear and

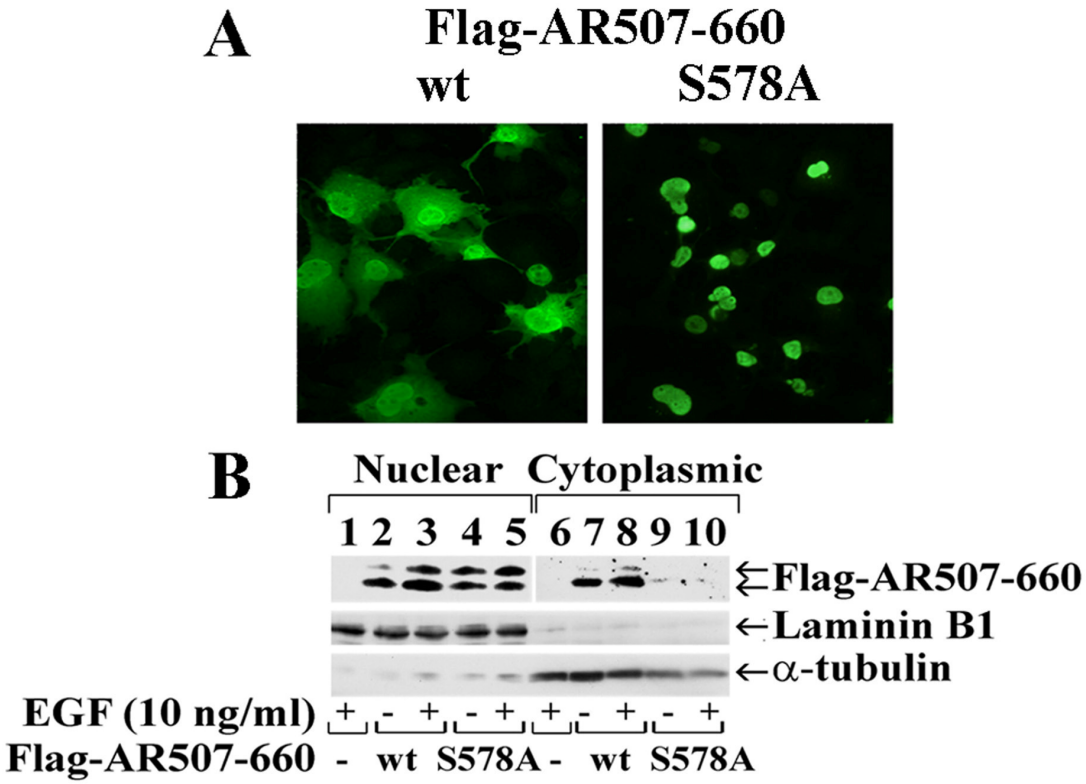

Figure 3.20. Increased nuclear localization of the AR S578A mutant. (**A**) COS cells were transfected with 0.2 µg wild-type (wt) Flag-AR-(507-660) or the S578A mutant and serum starved the next day for 24 h. Flag-AR-(507-660) and the S578A mutant are represented by green fluorescence detected using AR52 antibody and fluorescent-(FITC)-conjugated secondary anti-rabbit antibody. Original magnification 40X (**B**) Nuclear and cytoplasmic fractions were prepared as described under Experimental Procedures and analyzed by immunoblotting using AR52 antibody that recognizes Flag-AR-(507-660) wt and the S578A mutant. Laminin-B1 and α-tubulin served as nuclear and cytoplasmic extract controls, respectively.

cytoplasmic fractions, where the slower migrating Ser-515 phosphorylated form was prominent only in the nuclear fraction in response to EGF (Figure 3.20B, lanes 2, 3, 7 and 8). Flag-AR-(507-660)-S515A lacked the slower migrating form (see Figure 3.14) and distributed in both nuclear and cytoplasmic extracts similar to wild-type (data not shown). However, in agreement with the immunostaining results, Flag-AR-(507-660)-S578A was predominant only in nuclear extracts, with a greater proportion of the phosphorylated Ser-515 form (Figure 3.20B, lanes 4, 5, 9 and 10). Parallel immunoblotting of nuclear laminin-B1 and cytoplasmic α-tubulin substantiated the subcellular fractionation procedure. Cell extracts contained similar amounts of wild-type and mutant Flag-AR-(507-660) (data not shown), suggesting that the smaller amount of the S578A mutant in the cytoplasmic fraction did not result from degradation.

These results suggest that EGF dependent phosphorylation at AR Ser-578 limits nuclear phosphorylation at Ser-515 and modulates AR nuclear-cytoplasmic shuttling.

AR SER-578 PHOSPHORYLATION MODULATES THE AR INTERACTION WITH KU-70/80 INDEPENDENT OF AR BINDING TO DNA

The Ku-70/80 regulatory subunits of DNA-PK were shown previously to interact with the progesterone receptor DNA binding domain (31), which shares sequence similarity to the AR DNA binding domain (32). Ku-70/80 subunits were also implicated in AR transcriptional recycling (33). To address the influence of Ser-578 phosphorylation on the AR interaction with Ku-70/80, we performed coimmunoprecipitation studies in COS cells using full-length wild-type Flag-AR, the S578A mutant and endogenous Ku-70/80. We found that Ku-70 and Ku-80 coimmunoprecipitated with Flag-AR-S578A, but

only weakly with wild-type AR (Figure 3.21A, upper two panels). In similar experiments using Flag-AR-(507-660), we found that the S578A mutant interacted with endogenous Ku-70 and Ku-80 to a greater extent than wild-type Flag-AR-(507-660) in both COS (Figure 3.21B) and CWR-R1 cells (Figure 3.21C). Also, as seen with full-length Flag-tagged AR, the interaction between wild-type Flag-AR-(507-660) and Ku-70/80 increased in the response to EGF in both cell lines. A DNA binding mutant, Flag-AR-(507-919)-C576A, interacted with Ku-70/80 indicating the interaction is not dependent on AR/DNA binding (Figure 3.22).

These results suggest that EGF dependent phosphorylation at AR Ser-578 modulates phosphorylation at Ser-515 and regulates AR interaction with the Ku-70/80 regulatory subunits of DNA-PK.

INHIBITION OF AR-MEDIATED MMTV-LUC TRANSCRIPTION BY A NEGATIVE REGULATORY ELEMENT-1 (NRE-1)

Transient transfection in Ishikawa cells revealed AR-S578A did not activate MMTV-Luc (Figure 3.23) but it does transactivate PSA-Enh-Luc which lacks NRE-1. Ku-70/80 repressed GR transactivation through high affinity, sequence specific binding to double stranded NRE1 at -394 to -381 in the MMTV long terminal repeat (141,156). Repression of GR transactivation of the MMTV promoter correlated with recruitment of Ku-70/80 to NRE1 (141,157). To determine whether AR-mediated activation of MMTV –Luc is regulated by NRE-1, two MMTV-LTR deletion mutants were tested. Deletion mutant 1 lacked a sequence of NRE-1 (NREΔ-381 – -394) (MMTV–ΔNRE1-Luc) that is

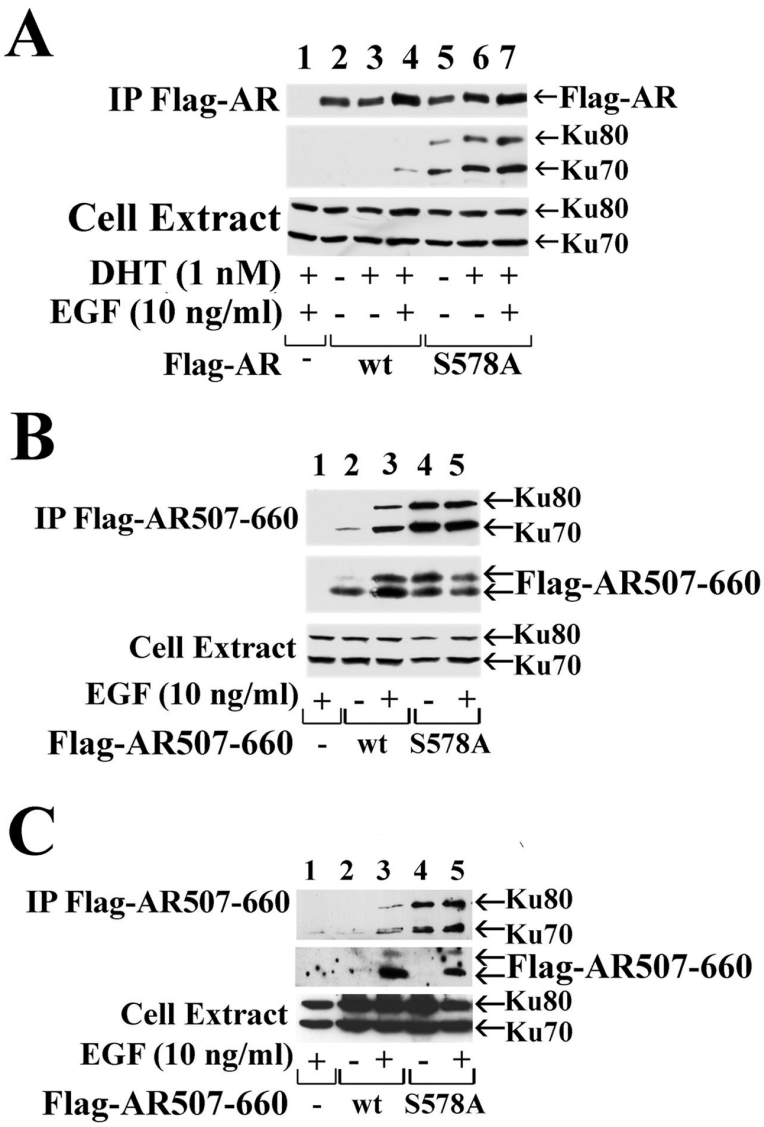

Figure 3.21. AR Ser-578 mediates the interaction with Ku-70/80. (**A, B**) COS cells and (**C**) CWR-R1 cells were transfected with 2 µg wild-type (wt) Flag-AR-(507-660) and the S578A mutant. Cells were serum-starved the next day for 24 h and treated with and without 1 nM DHT and/or 10 ng/ml EGF for 5 h as indicated. Protein lysates were immunoprecipitated (IP) using anti-Flag resin and analyzed by immunoblotting. AR-52 antibody was used to detect immunoprecipitated Flag-AR, and Ku-70/80 specific antibodies for coimmunoprecipitated proteins. Protein lysates (4% of total) were analyzed for endogenous Ku-70/80 (lower panels).

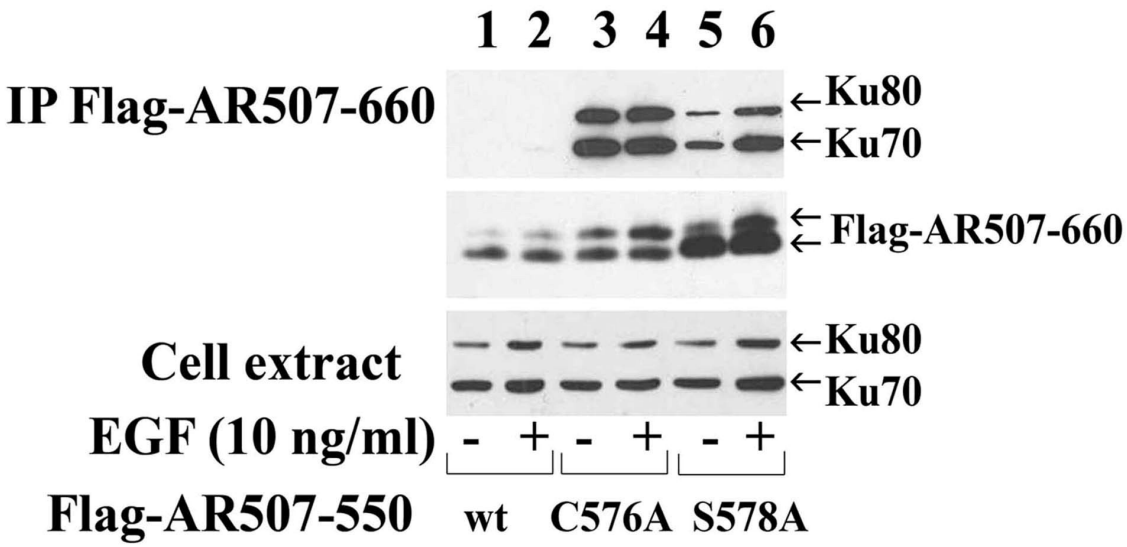

Figure 3.22. DNA-binding dependence of AR507-660 and Ku-70/80 interaction. COS cells were transfected with 2 μg wild-type (wt) Flag-AR-(507-660), C576A or S578A mutants. Cells were serum-starved the next day for 24 h and treated with and without 10 ng/ml EGF for 5 h as indicated. Protein lysates were immunoprecipitated (IP) using anti-Flag resin and analyzed by immunoblotting. AR-52 antibody was used to detect immunoprecipitated Flag-AR, and Ku-70/80 specific antibodies for coimmunoprecipitated proteins. Protein lysates (4% of total) were analyzed for endogenous Ku-70/80 (lower panels).

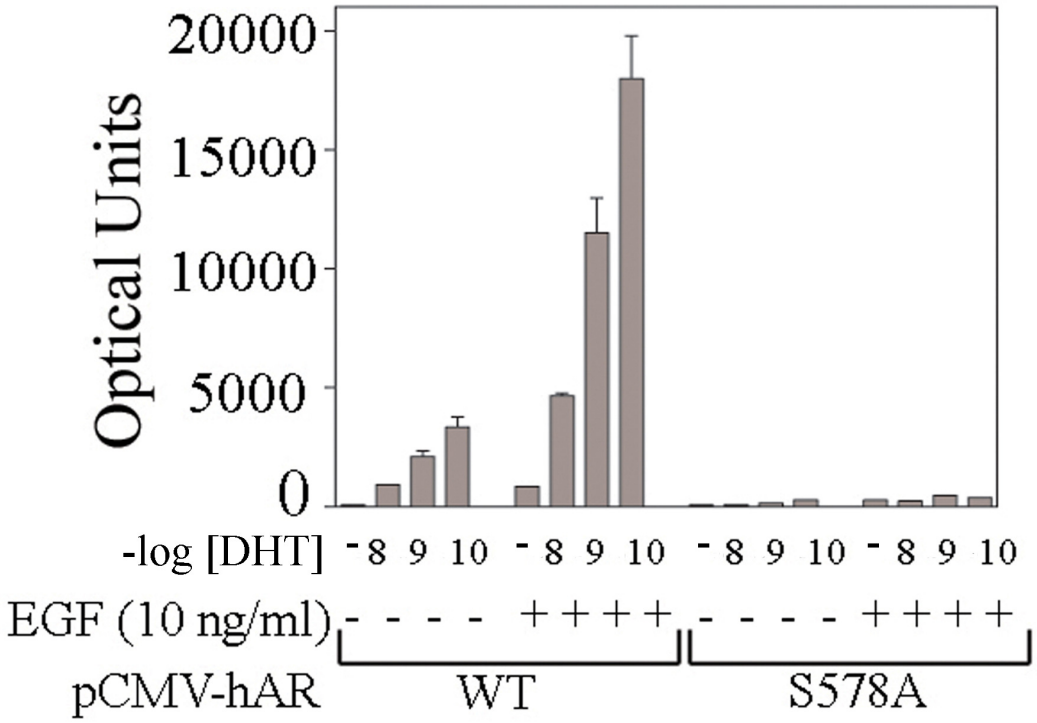

Figure 3.23. AR Ser-578 required for MMTV transcriptional activation in Ishikawa cells. Ishikawa cells were transiently transfected with 0.1 µg MMTV-Luc and 10 ng wild-type (WT) pCMV-AR and the S578A mutant and incubated for 24 h with and without increasing concentrations of DHT and 10 ng/ml EGF as indicated. Luciferase activity was determined the next day and are representative of 3 experiments.

conserved among other NRE-like sequences (141). Deletion mutant 2 lacked the NRE-1 region (NREΔ-421-364) (MMTV–ΔNRE2-Luc). CWR-R1 (Figure 3.24A) and Ishikawa (Figure 3.24B) cells were transfected with wild-type-MMTV-Luc, MMTV–ΔNRE1-Luc or MMTV–ΔNRE2-Luc reporter gene. In the absence of NRE-1 (-421– -364) there was a ~2 fold increase in the androgen-dependent transcriptional activation suggesting that NRE-1 functions as a regulatory element in MMTV-promoter transactivation by AR.

To test the hypothesis that phosphorylation at AR-Ser-578 decreases the interaction with Ku-70/80 to inhibit NRE-1-mediated down-regulation of MMTV transcription, pCMV-AR-S578A was cotransfected with MMTV-Luc or MMTV–ΔNRE2-Luc in CWR-R1 cells. Transcriptional activation of MMTV-Luc by AR-S578A was increased in MMTV–ΔNRE2-Luc. However, overall transcriptional activity by AR-578A was ~10-fold lower than wild-type AR, indicating that Ku70/80-mediated regulation is not sufficient to impair MMTV-Luc transcriptional activation by AR-S578A.

To determine whether NRE-1-mediated inhibition of MMTV activation by AR is modulated by Ku-70/80, XR-V15B cells were transfected with pCMV-AR and MMTV-Luc or MMTV–ΔNRE2-Luc (Figure 3.25). The results indicate that in Ku-deficient cells, deletion of NRE-1 increases AR activation of MMTV-Luc transcriptional activation by AR. Reconstituting AR by cotransfecting AR and Ku80 resulted in partial inhibition of MMTV transcription. This suggests that in the context of androgen signaling, inhibition of MMTV transcription has both Ku-70/80 dependent and independent pathways. Additional studies are required to determine the mechanisms of MMTV regulation by NRE1 in AR signaling pathways.

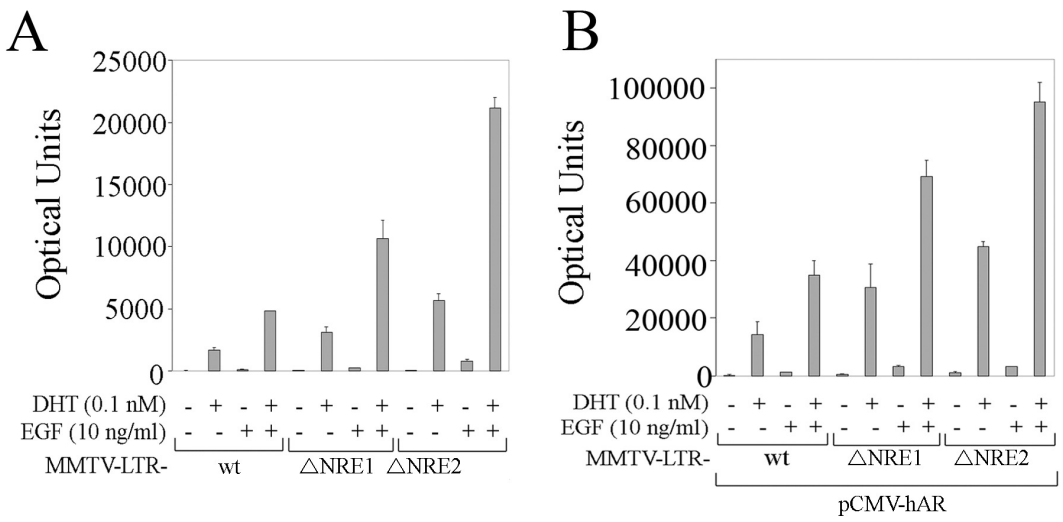

Figure 3.24. Reduced AR activation of MMTV-ΔNRE in CWR-R1 and Ishikawa cells. Cells were transfected with 0.1 µg MMTV-Luc wild-type (wt), ΔNRE1 (-381–-394), or ΔNRE2 (-421–-364) **(A)** alone in CWR-R1 or **(B)** with 25 ng pCMV-hAR in Ishikawa cells. Cells were incubated for 24 h with and without 0.1 nM DHT and 10 ng/ml EGF as indicated. Luciferase activity was determined the next day and are representative if two independent experiments.

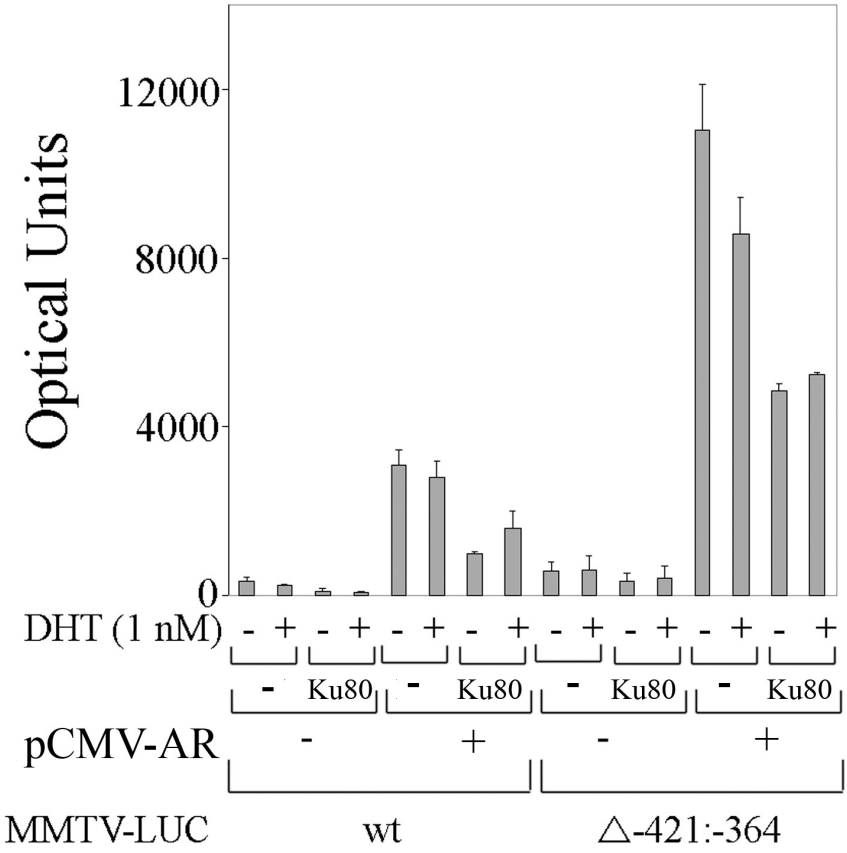

Figure 3.25. Ku80 and NRE-1-mediated inhibition of MMTV transcriptional activation by AR. XRV15B cells ($2.5x10^6$/6 com dish) were transiently transfected with 4 μg wild-type (wt) MMTV-Luc or MMTV-ΔNRE2 (Δ-381–-394), 1 μg pCMV-hAR and 2 μg empty vector pCDNA3.1 (-) or pCDNA-Ku80 (Ku80) as indicated. Cells were incubated for 24 h with and without 1 nM DHT and luciferase activity was determined the next day. The data are representation of two independent experiments.

DISCUSSION

EGF AND TIF2 IN CASTRATION- RECURRENT PROSTATE CANCER

Castration-recurrent prostate cancer expresses levels of AR similar to those found in androgen-stimulated prostate cancer and benign prostate. This observation suggests that the AR is important for growth regulation in castration-recurrent prostate cancer. Findings suggest prostate cancer that recurs during androgen deprivation therapy remains androgen dependent. Recent reports have demonstrated testosterone levels in castration-recurrent prostate cancer are sufficient to activate AR and up-regulate androgen response genes (76). Our studies have elucidated mechanisms by which growth factors increase AR activity in the presence of low levels of androgen (79,80).

EGF increased androgen-dependent AR transactivation in association with increased levels of TIF2 in the CWR-R1 cell line suggests p160 coactivators play a role in progression of prostate cancer. To investigate the function of EGF on TIF2 mediated increase of AR transcriptional activation in CWR-R1 cells, we explored the possibility of TIF2 mRNA up regulation by EGF. Our results demonstrated similar TIF2 mRNA levels in the absence and presence of EGF suggesting EGF does not play a regulatory role at the level of TIF2 transcription. Reducing TIF2/GRIP1 levels in CWR-R1 cells using

inhibitory RNAs resulted in a decreased AR transcriptional response to DHT and EGF. The data provide evidence for a direct link between AR transcriptional activity in recurrent prostate cancer, EGF signaling, and increased p160 coactivator levels. Data from our studies indicated increased AR transcriptional activity occurs in response to EGF in part from an increase in the androgen-dependent association between AR and TIF2 and that the EGF-induced increase in AR transactivation was linked to MAPK-mediated phosphorylation of TIF2 (79). Future studies addressing the clinical implications of EGFR and MAP kinase inhibitors on TIF2 expression and AR activity after androgen deprivation therapy are necessary to evaluate the biological significance of these pathways in castration recurrent prostate cancer. Xenograft studies in the absence and presence of androgen, EGFR and MAP kinase inhibitors would elucidate the dependence of CWR-R1 cell tumorogenic potential on EGF-mediated phosphorylation of TIF2.

EGF AND THE AR N/C INTERACTION

Transient expression of TIF2 in the presence of DHT and EGF had a stabilizing effect on AR F*XX*AA/A*XX*AA, an AR with mutations in the NH_2-terminal ^{23}FQNLF^{27} and ^{433}WHTLF^{437} sequences that are required for the androgen-induced AR N/C interaction (40,47,48,150,158). Mutations that cause loss of the N/C interaction allow greater accessibility of AF2 in the ligand binding domain to activation by p160 coactivators such as TIF2 (48,150). Surprisingly, whereas loss of the N/C interaction reduced AR transactivation of the PSA promoter in other cell lines (47), this mutant was as effective as wild-type AR when assayed in the CWR-R1 cell line, supporting the

notion that higher levels of TIF2 compensate for loss of the AR N/C interaction. DHT is thought to be the main modulator of AR dimerization but little is known about the effects of growth factors in this key regulatory event. Mammalian two hybrid interaction assays have been used extensively to study the AR N/C interaction and to determine the effects of EGF on AR interaction with TIF2 (40). In data not shown here, interaction of GAL4-AR1-503 with the AR-VP16 fusion vector increased in the presence of DHT and EGF as compared to DHT alone as demonstrated by the transcriptional activation of the 5XGAL4-Luc reporter. This suggests EGF plays a role in the AR N/C interaction and may modulate AR activity through mechanisms involving receptor dimerization. The EGF-induced increase in DHT-mediated N/C interaction was abrogated by VP16-AR-1-507-S308A indicating a link between EGF-mediated serine phoshorylation in the AR N-terminal domain and AR dimer formation. Future studies addressing AR-Ser-308 phosphorylation are important to determine the role of post-translational modifications in AR dimerization and the mechanism by which EGF drives AR function in castration recurrent prostate cancer. These studies could provide evidence to support the design of therapies targeting EGF signaling, the AR N/C interaction and kinases involved in promoting recurrence in the presence of low levels of androgen.

EGF REGULATION OF AR TRANSCRIPTIONAL ACTIVITY

EGF signaling has been indirectly linked to increased AR transcriptional activity through the post-translational modification of AR coregulatory proteins. EGF increases phosphorylation and multiple mono-ubiquitinylation of MAGE-11 of the melanoma antigen gene family (46). These changes stabilize the coregulator interaction with AR to

increase AR transcriptional activity. In our studies we provide evidence that EGF increases AR transcriptional activity through the coordinate phosphorylation of serine residues in the AR NH_2-terminal and DNA binding domains.

We have shown that EGF increases CWR-R1 prostate cancer cell growth in an AR dependent manner in the absence and presence of androgen. EGF acts synergistically with DHT to stimulate AR transcriptional activity and cell growth. EGF-dependent CWR-R1 prostate cancer cell proliferation was greatest in the presence of AR and DHT and was reduced by an inhibitor of PKC. One interpretation suggests that AR signaling in the absence and presence of DHT establishes a basal proliferation rate which is enhanced by EGF through mechanisms that include AR but are also independent of AR. EGF dependent AR activation in the absence and presence of androgen is mediated by PKC dependent phosphorylation at Ser-578 in the AR DNA binding domain and by MAP kinase dependent phosphorylation at Ser-515 in the AR NH_2-terminal region. The downstream functional effects of AR phosphorylation at these sites alter AR nuclear-cytoplasmic shuttling through interactions with Ku-70/80.

EGF-DEPENDENT AR PHOSPHORYLATION

Earlier mutagenesis studies demonstrated AR phosphorylation at Ser-81 and Ser-94 in the NH_2-terminal region, and at Ser-650 in the hinge region between the DNA and ligand binding domains (88). AR Ser-81 was suggested to be a substrate for the HER2 regulated kinase pathway (99) and AR phosphorylation at Ser-650 was linked to stress kinase modulation of AR transcriptional activity (159). However, mutations at these sites have relatively little effect on AR transcriptional activity (88,94). Mass spectrometry

confirmed AR phosphorylation at Ser-81, 94 and 650, and identified Ser-16, 256, 308 and 424 as phosphorylation sites in the AR NH_2-terminal region (85). AR NH_2-terminal Ser-213 was phosphorylated in nonproliferating prostate epithelial cells (89), and Akt mediated phosphorylation at AR Ser-213 and Ser-791 was linked to anti-apoptotic and proliferative effects in prostate cancer cells (86,160). However, the synergistic effects of Akt and AR on neoplastic proliferation of murine prostatic epithelium were independent of phosphorylation at these sites (161). More recently, prostate cancer growth was linked to Src and Ack1 mediated phosphorylation of AR tyrosine residues 267 and 363 in the AR NH_2-terminal region (95,162).

Our current studies focused on the AR DNA binding domain and flanking NH_2-terminal and hinge regions and confirmed that a mutation at Ser-650 has relatively little effect on AR transcriptional activity (88,94). We also found that mutations at other previously reported phosphorylation sites in the region, including a recently reported Src dependent tyrosine phosphorylation site Tyr-534 in the AR NH_2-terminal region close to the DNA binding domain (95), did not diminish AR transcriptional activity in CWR-R1 cells in response to DHT with or without EGF. The relative lack of functional effects of mutations at most of the previously reported AR phosphorylation sites left unanswered the question of the significance of AR phosphorylation, particularly in prostate cancer cells where growth factor and mitogen signaling are increased.

Our studies indicate direct effects of EGF signaling on AR transcriptional activity through Ser-515 in the NH_2-terminal region and Ser-578 in the DNA binding domain. MAP kinase phosphorylation site Ser-515 is positioned near the DNA binding domain (Figure 4.1A) carboxyl-terminal to AF1 transactivation domain residues 142-337 required for androgen dependent AR transcriptional activity (37,163). AR Ser-515 is

closer to tau-5 residues 360-485 (164), a transcriptional activation region whose activity is apparent in the AR NH_2-terminal and DNA binding domain fragment that lacks the ligand binding domain (37,163).

MAP kinase signaling was previously implicated in androgen dependent AR transcriptional activity (79,80,165). *In vitro* MAP kinase assays suggested phosphorylation of AR-36-643, an AR NH_2-terminal and DNA binding domain fragment, in response to HER2/neu signaling (107). Although an AR S515A mutation reduced the signaling effects of HER2/neu, additional evidence for phosphorylation at this site was not reported. Here we show that EGF signaling slowed the gel electrophoretic migration of an AR NH_2-terminal–DNA binding domain–hinge region fragment, an effect that was eliminated by treatment with λ-phosphatase. The EGF dependent increase in AR transcriptional activity was linked to phosphorylation at Ser-515 and Ser-578. MAP kinase consensus site mutation S515A reduced AR transcriptional activity slightly, but did not eliminate the AR transcriptional response to EGF in the absence and presence of androgen. Inhibition of Ser-578 phosphorylation by an S578A mutation increased phosphorylation at Ser-515, which provided evidence that AR phosphorylation at Ser-515 is linked to phosphorylation at Ser-578.

Indeed, EGF-dependent phosphorylation at AR Ser-578 appears to be required for EGF stimulation of AR transcriptional activity. Ser-578 is positioned in the first zinc module of the AR DNA binding domain (Figure 4.1B) within the P-box. Response element DNA binding specificity distinguished by the glucocorticoid receptor (GR) and estrogen receptor is associated with three P-box residues, GSxxV (116,166). Human AR P-box ^{577}GSCKV^{581} contains Ser-578 and is common to GR and the progesterone and mineralocorticoid receptors, each of which recognizes the GGTACAnnnTGTTCT

consensus response element (167-169). P-box squence within this subfamily distinguishes TGTTCT half-site sequence from TGACCT estrogen response element consensus sequence (166). Within the AR receptor subgroup, DNA response element binding specificity was attributed to residues in the second zinc module and the carboxyl-terminal extension in the hinge region (169,170).

Crystal structures of the AR DNA binding domain–androgen response element DNA complex indicate that human AR Ser-578 lies within the first alpha helix that directly contacts the major groove of the DNA (Figure 4.1 C) (171). Human AR Ser-578 corresponds to human GR Ser-440. When GR Ser-440 was changed to glycine to mimic Gly-204 in human ER or to alanine, GR DNA binding specificity was reduced. Based on these studies, the AR Ser-578 hydroxyl group is predicted to modulate DNA binding specificity through a hydrogen bond network extending to Arg-608 in the second zinc module loop containing AR D-box ^{596}ASRND^{600} (Figure 4.1B) (172). Unlike the common P-box sequence shared by AR, GR, progesterone and mineralocorticoid receptors, the AR D-box, thought to be involved in receptor dimerization and half site spacing recognition, differs from the AGRND D-box common to other members of this

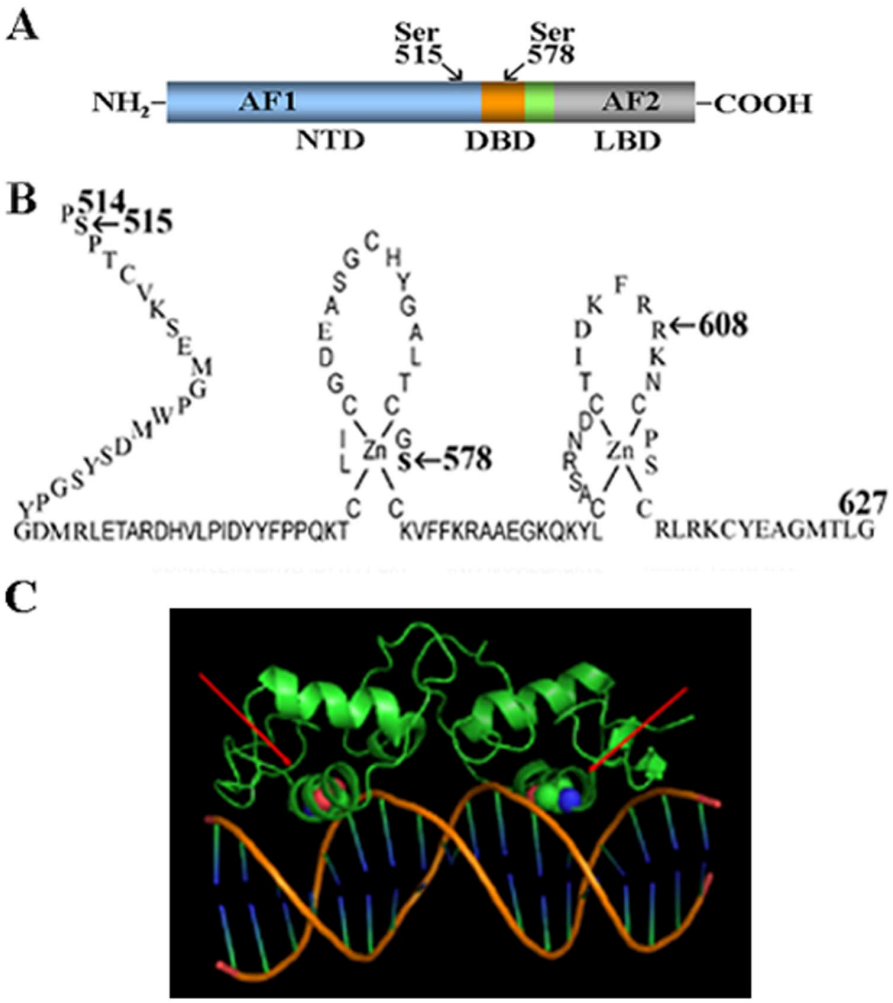

Figure 4.1. AR schematic diagram. **(A)** Full-length human AR amino acid residues 1-919 contain the NH_2-terminal domain (NTD) with activation function-1 (AF1) and Ser-515, DNA binding domain (DBD) with Ser-578, and ligand binding domain (LBD) with activation domain-2 (AF2). **(B)** AR NH_2-terminal and DNA binding domain residues 514-627 showing the two zinc modules with highlighted AR phosphorylation sites Ser-515 and Ser-578. (**C**) Structure of the AR DNA binding domain dimer (green) bound to androgen response element DNA (orange and blue) (52) with space filled Ser-578 indicated with red arrows.

subgroup of steroid receptors (115,116). AR Arg-608 corresponds to Arg-470 in human GR, for which a lysine substitution was predicted to alter DNA binding specificity by changing the hydrogen bonding pattern (172). The importance of AR Arg-608, which contacts the DNA phosphate backbone and is conserved in the steroid receptor family, is supported by the naturally occurring R608K mutation that causes partial androgen insensitivity (www.mcgill.ca/androgendb/).

A naturally occurring AR S578T mutation also causes grade 5 partial androgen insensitivity, where complete androgen insensitivity (grade 6/7) is associated with an external female phenotype in a genetic male (173,174). The S578T mutation demonstrates the importance of Ser-578 in AR function *in vivo*. The partial response of recombinant AR-S578T to elevated androgen levels in a COS cell transcriptional assay (174) indicates some retention of DNA binding activity. The extent of phosphorylation at residue 578 may be diminished by threonine at this position and phospho-Ser-578 or phospho-Thr-578 might alter AR interaction with DNA (Figure 4.1C) or associated proteins such as Ku-70/80. Rapid release and rebinding of steroid receptors to DNA in a dynamic hit-and-run model has revealed the transient nature of steroid receptor DNA binding required for activation of transcription (175,176). Similar transient interactions were reported for AR binding to androgen response element DNA (177). EGF dependent phosphorylation at AR Ser-578 may regulate AR association and dissociation from DNA and the magnitude of the transcriptional response.

We have provided evidence for PKC phosphorylation at AR Ser-578. Our results support previous evidence that phosphorylation by PKC in the DNA binding domain modulates nuclear receptor localization. PKC was shown to phosphorylate highly conserved Ser-78 between the two zinc modules in the DNA binding domain of

hepatocyte nuclear factor-4α (HNF-4α), and at a corresponding position in retinoic acid receptor-α, retinoid X receptor-α and thyroid hormone receptor-α (60). HNF-4α Ser-78 is conserved through most of the nuclear receptor superfamily, including Ser-212 in human estrogen receptor-α. However, the corresponding residue in the AR subgroup of steroid receptors is alanine and is Ala-586 in human AR. PKC phosphorylation at Ser-78 in the DNA binding domain of HNF-4α and other nuclear receptors regulates nuclear localization (178). HNF-4α-S78A bound DNA, but the S78D phosphomimetic did not. The AR phosphomimetic S578D retained greater transcriptional activity than AR-S578A, but less than wild-type AR (data not shown), and like wild-type AR, the S578D mutant was distributed in both the cytoplasm and nucleus. AR-S578D might be expected to bind less Ku-70/80, which could interfere with the dynamic model of steroid receptor binding to DNA.

Phosphorylation in the DNA binding domain of different nuclear receptors has been involved with changes in the regulation of gene transcription. Black et al. (179) demonstrated that mutations in the AR DBD ΔF582 and F582Y and the export mutant F582,573A displayed androgen-dependent arrest in foci were steroid receptor coactivator glucocorticoid receptor-interacting protein (GRIP)-1 is highly expressed. The data suggest that wild-type AR induces rapid, agonist dependent dissociation of GRIP-1 from subnuclear foci, which may facilitate interactions between nuclear receptors and coactivators before transcription. The AR-C619Y mutant, which alters a cysteine that coordinates zinc in the AR DNA binding domain, was transcriptionally inactive and unable to bind DNA and localized abnormally in nuclear aggregates that also contain SRC-1, suggesting that this mutant may alter cellular physiology through sequestration of

critical proteins (180). This data agrees with our observation that modifications on the AR DBD regulate AR localization and interactions with coregulators.

Studies indicate there is convergence between the PKC and androgen-mediated pathways. Analysis of the *PKC* δ promoter revealed a putative androgen responsive element (ARE) located 4.7 kb upstream from the transcription start site and luciferase reporter assays show that this element is responsive to androgens (93). Crosstalk between PKC and other kinases was demonstrated by studies using phorbol 12-myristate 13-acetate which activates p38 MAPK in LNCaP cells the induction of apoptosis (181). Its been shown that the basal level of PKCε protein is elevated in recurrent CWR22 xenograft tumors and subconfluent cultures of androgen independent prostate cancer cell lines (DU145 and PC-3), relative to androgen-sensitive prostate cancer cell lines (CWR22 tumors and LNCaP cells) (182).

The results raise the possibility that phosphorylation by PKC at conserved serine residues in the first zinc module of the DNA binding domain regulates DNA binding for the entire nuclear receptor family. Phosphorylation within the DNA binding domain may be a common regulatory mechanism that controls nuclear-cytoplasmic shuttling and DNA binding required for differential gene regulation.

AR NUCLEAR-CYTOPLASMIC SHUTTLING AND DNA BINDING SPECIFICITY

EGF-dependent AR transcriptional activity mediated by PKC-dependent phosphorylation at AR Ser-578 is linked to AR nuclear-cytoplasmic shuttling. Based on results with an AR NH_2-terminal, DNA binding and hinge region fragment, where the S578A mutant was exclusively nuclear, EGF dependent AR phosphorylation at Ser-515

and Ser-578 modulates AR nuclear retention. In addition, AR coimmunoprecipiates with Ku-70/80, a DNA binding protein complex that regulates DNA-PK activity and other transcription factors (183). The greater nuclear retention of AR-(507-660)-S578A compared to wild-type was associated with increased interaction with Ku-70/80. PKC mediated AR phosphorylation at Ser-578 appears to regulate the AR interaction with Ku-70/80 and modulate AR nuclear-cytoplasmic shuttling and DNA binding. Our studies are in agreement with previous evidence that steroid receptor phosphorylation is linked to nuclear-cytoplasmic shuttling (184).

In agreement with evidence that AR-S578A interacts to a greater extent with Ku-70/80, full-length AR-S578A did not activate MMTV-Luc in Ishikawa cells (data not shown), even though AR-S578A transcriptional activity was similar to wild-type in Ishikawa cells using the PSA-Enh-Luc reporter which lacks a negative response element (NRE) sequence present in the MMTV promoter-enhancer. Ku-80 represses GR transactivation through high affinity, sequence specific binding to double stranded NRE1 at -394 to -381 in the MMTV long terminal repeat (141,156). Repression of GR transactivation of the MMTV promoter correlated with recruitment of Ku-70/80 to NRE1 (141,142,157).

Loss of AR-S578A transactivation of MMTV-Luc did not result from inhibition of AR binding to DNA. In the context of the AR NH_2-terminal and DNA binding domain constitutively active fragment, AR-S578A retains wild-type activity with MMTV-Luc (data not shown). This is consistent with a previous study suggesting that Ku-70/80 interacts preferentially with the AR ligand binding domain and provides further evidence that the effect of the S578A mutation is to increase AR interaction with Ku-70/80. A study demonstrated nuclear AR was transcriptionally active on prostate-specific antigen

and MMTV promoters driving reporter genes. AR forced to the cytoplasm was largely inactive on the prostate-specific antigen promoter, but AR was active on the mouse mammary tumor virus promoter and on two endogenous genes examined (185). Our studies suggest that a stronger interaction between AR and Ku-70/80 is associated with loss of AR activation of MMTV. In addition, the results support the concept that EGF dependent phosphorylation at Ser-578 modulates AR transactivation through its interaction with Ku-70/80.

EGF-dependent phosphorylation sites Ser-515 and Ser-578 in AR differ from the GR DNA-PK phosphorylation site that was linked to GR association with Ku-70/80. Human GR hinge region DNA-PK phosphorylation site Ser-508 implicated in GR nuclear retention (142,156,186) is positioned close to human GR nuclear targeting residues 479-498, and displaced in position by two residues to the corresponding human AR hinge residue Ser-650 near AR nuclear targeting residues 617-633 (158,187). We and others have shown that a mutation at Ser-650 has relatively little effect on AR transcriptional activity (88,94). However, human AR has a DNA-PK consensus phosphorylation site ^{656}TQ^{657} in the hinge region and inhibition of DNA-PK was reported to reduce AR phosphorylation and nuclear export (136). Given that AR interacts with Ku-70/80, an interaction that is enhanced by the S578A mutation, and that Ku-70/80 has additional functions independent of DNA-PK (157), Ku-70/80 may regulate AR independent of DNA-PK. Ku-70/80 is reported to have DNA helicase activity (188) which may more directly influence AR transcriptional activity.

AR FUNCTION IN CASTRATION-RECURRENT PROSTATE CANCER

AR is an important transcriptional activator in castration-recurrent prostate cancer growth that follows a period of remission in response to androgen deprivation therapy. The AR gene is amplified in approximately one third of prostate cancers (189). AR somatic gene mutations in prostate cancer can increase AR transactivation by androgens and other ligands. However, although AR mutations are relatively common in established prostate cancer cell lines, they are infrequent in prostate cancer tissue specimens and cannot account for the high incidence of castration-recurrent prostate cancer growth after androgen deprivation therapy. AR somatic mutations in prostate cancer, such as AR-H874Y in the CWR22 human prostate xenograft and the derived CWR-R1 prostate cancer cell line used in the present study (66,69), tend to increase AR sensitivity to ligand dependent transactivation through more efficient recruitment of SRC/p160 coactivators by AF2 in the ligand binding domain (52). In contrast, ligand-independent AR activation may be independent of AF2 in the ligand binding domain (190), mediated instead by growth factor and mitogen downstream signaling mechanisms (191). This is supported by the inability of flutamide to inhibit castration-recurrent prostate cancer cell growth (192).

Chromatin studies support AR function in cell proliferation in castration-recurrent prostate cancer in the absence of androgen (193-197). Adeno-associated viral siRNA knockdown of AR diminished both DHT and EGF stimulated CWR-R1 prostate cancer cell growth. However, residual levels of AR in the AR-siRNA-scAAV treated cells appear to be sufficient to mediate prostate cancer cell growth in response to the synergistic effects of DHT and EGF, a finding relevant to clinical prostate cancer since androgens are present in castration-recurrent prostate cancer tissue (75). In addition, EGF was shown to induce site specific phosphorylation and mono-ubiquitinylation of AR coregulator MAGE-11 required to interact with AR and increase transcriptional activity

in the absence and presence of androgen (198). Thus, growth factor and mitogen signaling through multiple mechanisms that involve AR may account for the relapse of prostate cancer growth.

FUTURE PERSPECTIVES

PKC AND PROSTATE CANCER

We explored the role of PKC on AR DBD phosphorylation utilizing the catalytic subunit of PKC, which is conserved between all isoforms. We demonstrated that PKC phosphorylates AR at Ser-578 to possibly allow transient nuclear localization of and interaction with coregulators Ku-70/80 from the DNA-PK trimeric complex. Our data indicates PKC-mediated phosphorylation may contribute to EGF activation of AR in androgen independent environments by promoting optimal nuclear/cytoplasmic trafficking and dissociation from nuclear coregulators.

PKCα is necessary for EGFR signaling to ERK1/2 activation induced by phorbol esters in androgen-independent human prostate cancer PC-3 cells (199). However, among the PKC isozymes present in human prostatic epithelial cells, PKCε is implicated in counterbalancing the influence of PKCα and PKCδ by favoring a program that results in the malignant progression of prostate cancer (182). However, there is little clinical evidence at present to support a role for PKC in the development of castration recurrent prostate cancer and there is no clinical data to date demonstrating improved therapeutic outcome in response to PKC inhibition. Studies addressing PKC isoform expression and

activity during prostate cancer progression are required to determine if the PKC pathway a suitable therapeutic target for castration recurrent prostate cancer.

RECURRENT PROSTATE CANCER AND EGF-MEDIATED SIGNALING

Analyses have been performed by others to determine how nucleocytoplasmic shuttling contributes to the function of full-length AR utilizing heterokaryon assays in COS cells (185). It is yet to be determined whether EGF-dependent phosphorylation of AR-Ser-578 by PKC regulates full-length AR nuclear export. We examined the role of AR-Ser-578 phosphorylation by studying cellular localization of Flag-AR-(507-660) 5 h after EGF treatment. A more detailed look analyzing a larger range of EGF treatment time-points and concentrations as well as implementing the heterokaryon assay approach would help determine if the status of Ser-578 influences full-length AR cellular localization. Further, cells transformed with wild-type AR and AR-Ser-578 may provide a direct link between EGF, AR, PKC and CWR-R1 cell proliferation. Specifically, CWR-R1 cells where AR is inhibited could be reconstituted with knock-down resistant versions of the wild type AR or AR-S578A mutant. This requires the generation of a stable cell line by either selecting transformed clones or introducing AR-Ser-578 expression via a lentiviral vector. Cell proliferation assays with these AR-rescue cells may provide a direct test of the importance of AR-Ser-578 phosphorylation for the EGF-stimulation of cell proliferation. Furthermore, cells expressing AR-S578A could be used *in vivo* to test their tumorgenecity in nude mice xenografts to determine if phosphorylation at AR-Ser-578 inhibits castration recurrent cell growth in testicular androgen-depleted environments.

DNA-PK AND PROSTATE CANCER

This study provided a functional link between Ku-70/80 and AR. It is yet to be determined clinically if Ku-70/80 expression, cellular localization and interaction with AR plays a role in prostate cancer cell growth. It has been suggested that down-regulation of Ku-70/80 promotes progression of urothelial carcinogenesis to a more malignant and aggressive clinical behavior, presumably as a result of an impaired capacity for DNA repair (200). Furthermore, Ku-70 siRNAs induced a decrease in the surviving fraction of gamma irradiated human cervical epithelioid (HeLa) cells and similar sensitizing effects were observed for etoposide, a topoisomerase II inhibitor (201). A study investigated the Ku70/80 DNA-binding activity in human breast and bladder normal tissue and tumor biopsies. The tumor tissues revealed the existence of two different patterns in Ku-70/80 DNA-binding activity, overall correlated to the progression of the neoplastic disease. Breast and non-invasive bladder tumors showed a high nuclear DNA-binding activity. Conversely, all the corresponding normal tissues displayed a very low basal level of the DNA-binding activity. There are no clinical studies that correlate Ku-70/80 expression or cellular localization to sensitization to chemotherapy in prostate cancer patients. Analogous studies in the prostate are required to determine if Ku-70/80 activity after androgen deprivation influences AR function in the absence of testicular androgens and if targeting Ku-70/80 during recurrence is an effective mechanism for sensitization to chemotherapy.

CLINICAL IMPLICATIONS

A major goal of this study was to provide evidence for the role of AR in castration recurrent prostate cancer. We provided evidence that AR-Ser-578 mediates EGF-dependent activation of AR and plays a role in the DHT and EGF-mediated effects of AR transcriptional activation of the PSA-Enh-Luc reporter in CWR-R1 cells. It is yet to be determined if phosphorylation status of AR-Ser-578 is clinically relevant during evasion of androgen deprivation therapy. Immunohistochemistry of benign prostate, androgen-dependent, and recurrent prostate cancer with the newly developed phospho-AR-Ser-578 antibody would provide insight to whether Ser-578 phosphorylation contributes to prostate cancer recurrence. This may be a challenging goal because the newly-generated phosphospecific antibody requires optimization for immunoblotting and immunostaining. However, it would be clinically relevant to determine if the phosphorylation status of AR-Ser-578 correlates to progression towards prostate cancer recurrence. Furthermore, the DNA-PK consuensus site on AR ^{656}TQ^{657} was not analyzed in this study. Mutating this site and performing functional assays would be important to determine if DNA-PK phosphorylation plays a role in AR-mediated signaling in CWR-R1 cells.

scAAV vectors have been shown to deliver hairpin siRNA into multidrug-resistant human breast cancer cells and oral cancer cells (202). Furthermore, the feasibility of various scAAV serotypes as efficient gene delivery vehicles in human cancer cells was recently evaluated. Substantial transgene expression lasted over 30 days in various cancer cells lines following gene delivery, indicating that long-term gene expression can occur (203). Thus, there is evidence suggesting that scAAV vectors are suitable gene transfer tools potentially applicable to a wide variety of human cancer cells, independently driving persistent transgene expression. scAAV-ARsiRNA inhibits AR

expression in culture up to 5 days after infection. Optimization of delivery, uptake, and transduction efficiency *in vivo* of scAAV-ARsiRNA may elucidate if scAAV-mediated decrease of AR protein is a suitable tool for inhibiting castration-recurrent prostate cancer cell growth.

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