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INTRODUCTION

The androgen receptor (AR) is required for normal prostate development and the onset and progression of prostate cancer. AR has the modular structure characteristic of steroid hormone receptors, with an NH_2-terminal transcriptional activation domain, conserved DNA binding domain, hinge region and carboxyl-terminal ligand binding domain (1, 2). AR mediates the biological effects of androgens by binding testosterone and dihydrotestosterone (DHT) with high affinity (3). Androgen binding in the ligand binding domain stabilizes AR through the NH_2- and carboxyl-terminal N/C interaction that increases AR transcriptional activity (4). Androgen deprivation by surgical or chemical castration to treat advanced prostate cancer reduces AR transcriptional activity and promotes tumor regression.

Several mechanisms have been proposed to explain the emergence of castration-recurrent prostate cancer after androgen deprivation therapy (reviewed in ref. 5). AR transcriptional activity and CWR-R1 human prostate cancer cell proliferation are hypersensitive to DHT (6). AR localizes in nuclei of prostate cancer cells despite low levels of circulating androgen and appears to mediate recurrent growth after androgen deprivation (7). This could be explained by the presence of sufficient testosterone or DHT to activate AR in the microenvironment of castration-recurrent prostate cancer tissue (8, 9). On the other hand, cell culture studies suggest that AR transcriptional activity involves growth factor signaling under conditions of androgen deprivation.

HER2/neu, keratinocyte growth factor, insulin-like growth factor-1 and Il-6 have been reported to activate AR in the absence of androgen (10-12). Epidermal growth factor (EGF) dependent phosphorylation of transcriptional intermediary factor 2 and heregulin signaling through the HER2 and HER3 tyrosine kinases increase AR transactivation and alter the growth of CWR-R1 prostate cancer cells in response to low levels of androgen (13, 14). HER2 and HER3 activation, possibly through autocrine signaling, contributes to cell proliferation during prostate cancer recurrence (13). Growth factor signaling contributes to the onset of castration-recurrent prostate cancer through cross-talk between AR and autocrine loops that drive prostate cancer growth in the androgen deprived patient.

Despite evidence for AR transcriptional activity in castration-recurrent prostate cancer, there is little consensus regarding the mechanisms involved in growth factor mediated AR phosphorylation (15). The present study determined the effects of EGF on AR phosphorylation, nuclear localization and gene transactivation in prostate cancer cell proliferation.

BODY

AR Dependent CWR-R1 Prostate Cancer Cell Proliferation–We investigated the requirement for AR in castration-recurrent prostate cancer cell growth using the CWR-R1 cell line that was derived from the castration-recurrent CWR22 xenograft of human prostate cancer (6). Growth studies were performed using CWR-R1 cells infected with control or AR targeted siRNA-scAAV in the absence and presence of DHT and/or EGF. AR- siRNA-scAAV infected cells grew more slowly than control cells in the absence and presence of 0.1 nM DHT (Fig. 1A). The > 90% reduction in AR levels in CWR-R1 cells treated without (Fig. 1B, upper panel) and with DHT (Fig. 1B, 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.

EGF also stimulated control CWR-R1 cells to grow faster than cells infected with AR-siRNA-scAAV (Fig. 2A, 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 (Fig. 2A, 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 (Fig. 2B, 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 (Fig. 2B, right panel).

Androgen Independent AR transactivation–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 and presence of DHT. As expected, DHT increased AR transcriptional activity ~100 fold, with a further 3 fold increase after addition of EGF (Fig. 3A). 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 (Figs. 3A and 3B, lane 5). However, AR targeted siRNA oligonucleotide-3 that reduced AR levels (Fig. 3B, 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. 3A). 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 (Fig. 3C).

The results indicate that EGF can activate AR in the 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.

EGF Dependent AR Phosphorylation–Sequence analysis using NetPhos 2.0 (16) indicates 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 (Fig. 4A). Immunoblots of wild-type Flag-AR-(507-660) expressed in COS cells revealed a 27 kDa protein, and after treatment with EGF, an additional 29 kDa band (Fig 4B, lanes 1–3). The EGF dependent 29 kDa band was eliminated by treatment with λ-phosphatase in the absence, but not in the presence of phosphatase inhibitors (Fig. 4B, lanes 3–8). The 29 kDa band was also observed in response to EGF with Flag-AR-(507-660)-C576A, a mutant with a first zinc module cysteine mutation that eliminates DNA binding (data not shown). The appearance of an EGF dependent, phosphatase sensitive higher molecular weight form of Flag-AR-(507-660) indicated that EGF induces phosphorylation at one or more sites between AR residues 507-660 in a manner independent of AR binding to DNA.

To identify the EGF dependent 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 Fig. 4A. Immunoblot analysis of cell extracts before and after treatment with EGF indicated that only the AR NH_2-terminal MAP kinase consensus site mutation S515A eliminated the slower migrating form of Flag-AR-(507-660) when assayed in COS (Fig. 4C, upper panel, lanes 1–4) and CWR-R1 cells (Fig. 4C, lower panel, lanes 1–4). We found in addition that the PKC consensus site mutation S578A in the AR DNA binding domain increased the proportion of the slower migrating form attributed to phosphorylation at Ser-515 in the presence of EGF (Fig. 4C, lanes 5-6).

The results suggest that the EGF dependent increase in AR transcriptional activity and CWR-R1 cell growth shown above is associated with AR phosphorylation at MAP kinase consensus site Ser-515 in the NH_2-terminal region, and modulated by PKC consensus site Ser-578 in the DNA binding domain.

AR Phosphorylation Alters Transcriptional Activity–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 (Fig. 5A, 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 (17,18). 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 (Fig. 5A, 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 interaction (4,19).

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 (Fig. 5B, upper panel). However, similar to results with full-length AR-S578A shown in Fig. 5A, transcriptional activity of AR-(1-660)-S578A did not increase in response to EGF, even though expression levels of AR-(1-660)-S578A were similar to wild-type AR-(1-660) (Fig. 5B, 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 (Fig. 5C).

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

Functional effects of AR Phosphorylation by PKC –Ser-578 is a predicted consensus phosphorylation site for PKC, a kinase that acts downstream of EGF signaling (20). 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 (Fig. 6A, lanes 1 and 2). When averaged over multiple experiments, the S578A mutation decreased GST-AR-(1-660) phosphorylation by ~50% (Fig. 6B). The EGF dependent increase in CWR-R1 cell growth (Fig. 6C) was reduced in the presence of 50 nM Calphostin, a PKC specific inhibitor.

The 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 (Fig. 7A, 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 (Fig. 7A, 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 cytoplasmic fractions, where the slower migrating Ser-515 phosphorylated form was prominent only in the nuclear fraction in response to EGF (Fig. 7B, lanes 2, 3, 7 and 8). Flag-AR-(507-660)-S515A lacked the slower migrating form (see Fig. 4) 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 (Fig. 7B, 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.

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

Phosphorylation at AR Ser-578 Modulates AR Interaction with Ku-70/80– The Ku-70/80 regulatory subunits of DNA-PK were shown previously to interact with the progesterone receptor DNA binding domain (21), which shares sequence similarity to the AR DNA binding domain (22). Ku-70/80 subunits were also implicated in AR transcriptional recycling (23). 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 (Fig. 8A, 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 (Fig. 8B) and CWR-R1 cells (Fig. 8C). 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 to a similar extent as wild-type (data not shown).

The 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.

KEY RESEARCH ACCOMPLISHEMENTS

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. 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. 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.

REPORTABLE OUTCOMES

A manuscript entitled “Site specific androgen receptor serine phosphorylation linked to epidermal growth factor dependent growth of castration-recurrent prostate cancer” was recently published as an Epub ahead of print in the *Journal of Biological Chemistry*.

CONCLUSIONS

We have demonstrated that AR is required for CWR-R1 prostate cancer cell growth in response to androgen or EGF, and that DHT and EGF act synergistically to increase cell growth. EGF dependent phosphorylation at MAP kinase consensus site Ser-515 in the AR NH_2-terminal domain and protein kinase C consensus site Ser-578 in the AR DNA binding domain regulate AR nuclear-cytoplasmic shuttling through interactions with the Ku-70/80 regulatory subunits of DNA dependent protein kinase (DNA-PK). The studies suggest that EGF dependent AR phosphorylation in the P-box of the DNA binding domain first zinc module regulates AR transactivation in response to mitogen signaling. 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.

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Appendix I

FIGURE LEGENDS

Figure 1. 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).

Figure 2. 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. Inhibition of DHT and/or EGF stimulated AR transcriptional activity using AR siRNA. 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 in the absence or presence of 10 ng/ml EGF (**A**) or in the absence and presence of EGF alone (**C**) 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).

Figure 4. EGF dependent phosphorylation at AR Ser-515. (**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). (**C**) 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. (**D**) 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 5. 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".

Figure 6. AR Ser-578 required for EGF-induced AR transactivation in CWR-R1 and Ishikawa cells. (**A**) CWR-R1 cells were transiently transfected with 0.1 µg PSA-Enh-Luc and 10 ng wt pCMV-AR and the S578A, S515A, S515A-S578A, S650A and S578A-S650A mutants, and incubated for 24 h with and without 0.1 nM DHT and 10 ng/ml EGF as indicated and luciferase activity was determined (upper panel). COS cells were transfected using DEAE dextran as described under "Experimental Procedures" with 2 µg pCMV5 empty vector (-), wild-type pCMV-AR (wt) and the S578A and S515A mutations (lower panel). COS cells were incubated in the absence and presence of 10 nM DHT and 10 ng/ml EGF as indicated and immunoblots were performed to confirm similar expression levels. (**B**) CWR-R1 cells were transfected with 0.1 µg PSA-Enh-Luc and 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-AR-(1-660) and the S578A mutant and incubating cells for 24 h in the absence and presence of 10 ng/ml EGF (lower panel). (**C**) 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 7. 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)** 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.

Figure 8. 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.

Figure 9. 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 10. 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.

APPENDIX II

FIGURES

Figure 1

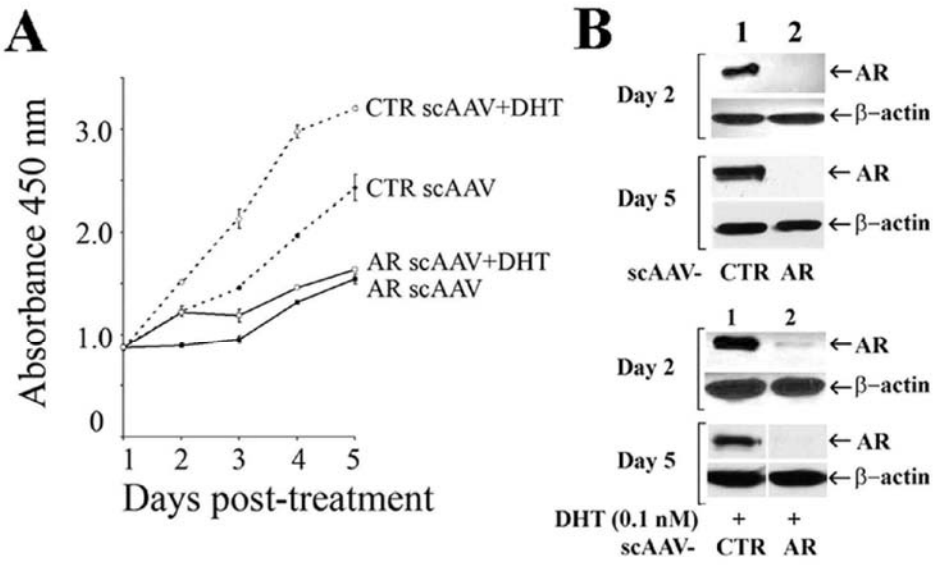

A
Absorbance 450 nm
3.0
2.0
1.0
0
1 2 3 4 5
Days post-treatment
CTR scAAV+DHT
CTR scAAV
AR scAAV+DHT
AR scAAV
B
1 2
Day 2
←AR
←β−actin
Day 5
←AR
←β−actin
scAAV- CTR AR
1 2
Day 2
←AR
←β−actin
Day 5
←AR
←β−actin
DHT (0.1 nM) + +
scAAV- CTR AR

Figure 2

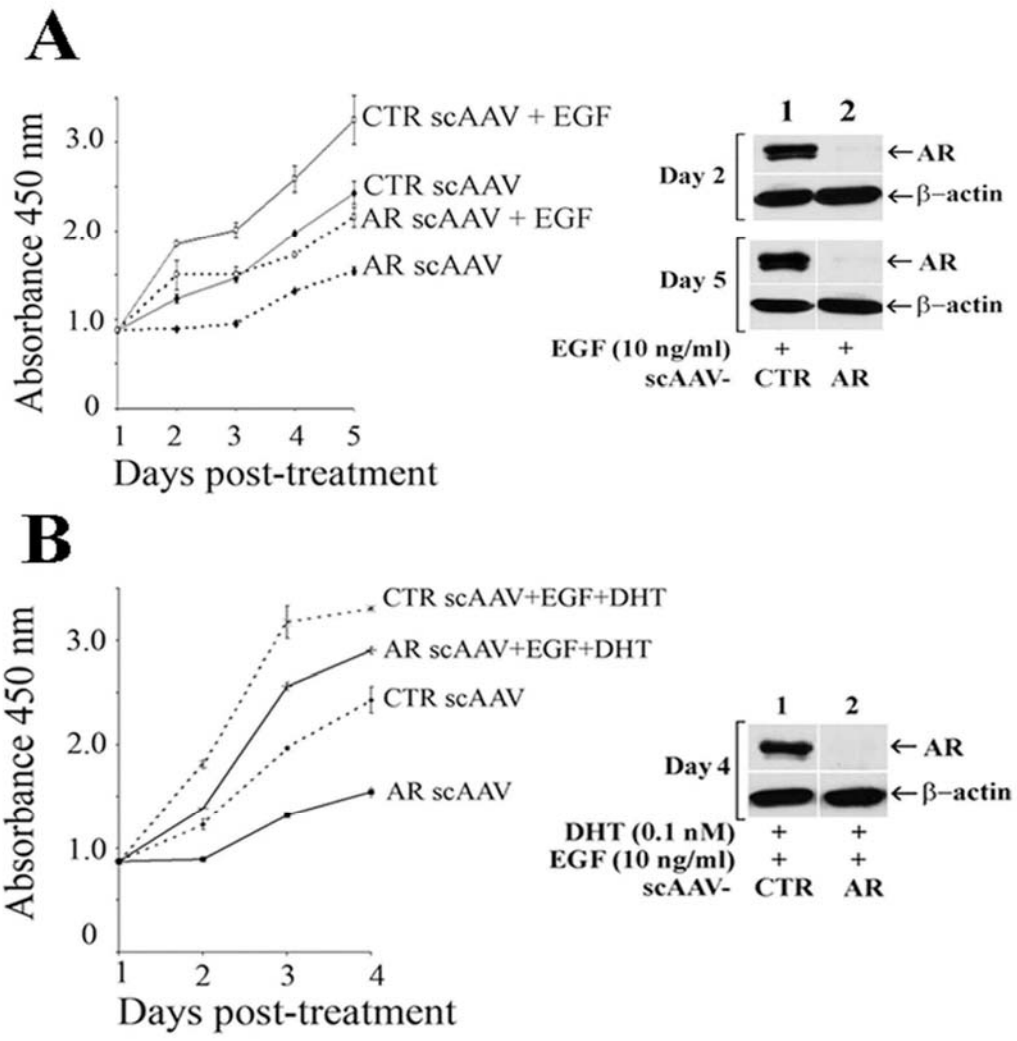

A
Absorbance 450 nm
3.0
2.0
1.0
0
1 2 3 4 5
Days post-treatment
CTR scAAV + EGF
CTR scAAV
AR scAAV + EGF
AR scAAV
1 2
Day 2
Day 5
←AR
←β–actin
EGF (10 ng/ml) + +
scAAV- CTR AR
B
Absorbance 450 nm
3.0
2.0
1.0
0
1 2 3 4
Days post-treatment
CTR scAAV+EGF+DHT
AR scAAV+EGF+DHT
CTR scAAV
AR scAAV
1 2
Day 4
←AR
←β–actin
DHT (0.1 nM) + +
EGF (10 ng/ml) + +
scAAV- CTR AR

Figure 3

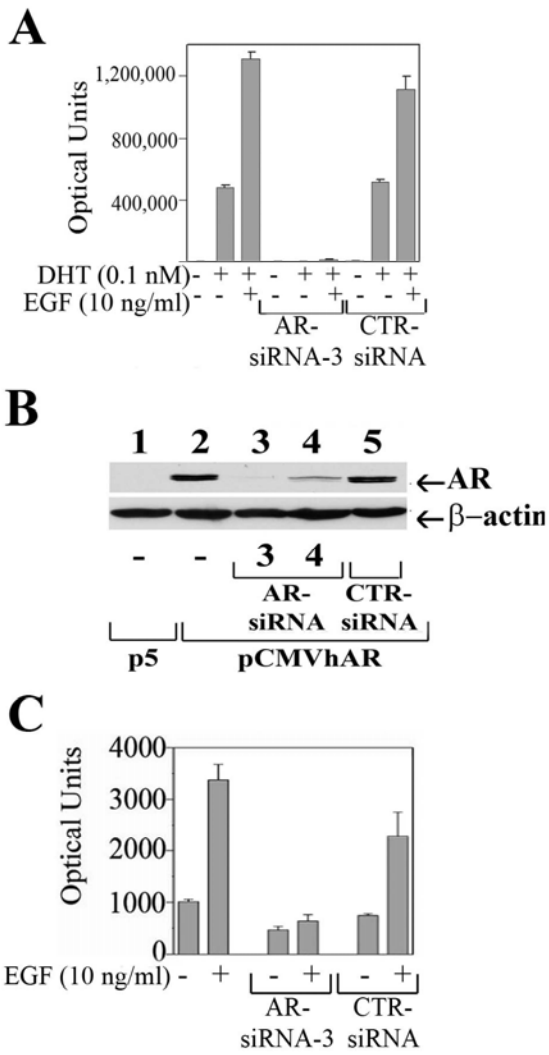

A
Optical Units
1,200,000
800,000
400,000
DHT (0.1 nM)- + + - + + - + +
EGF (10 ng/ml)- - + - - + - - +
AR-siRNA-3
CTR-siRNA
B
1 2 3 4 5
←AR
←β−actin
- - 3 4
AR-siRNA
CTR-siRNA
p5
pCMVhAR
C
Optical Units
4000
3000
2000
1000
0
EGF (10 ng/ml) - + - + - +
AR-siRNA-3
CTR-siRNA

Figure 4

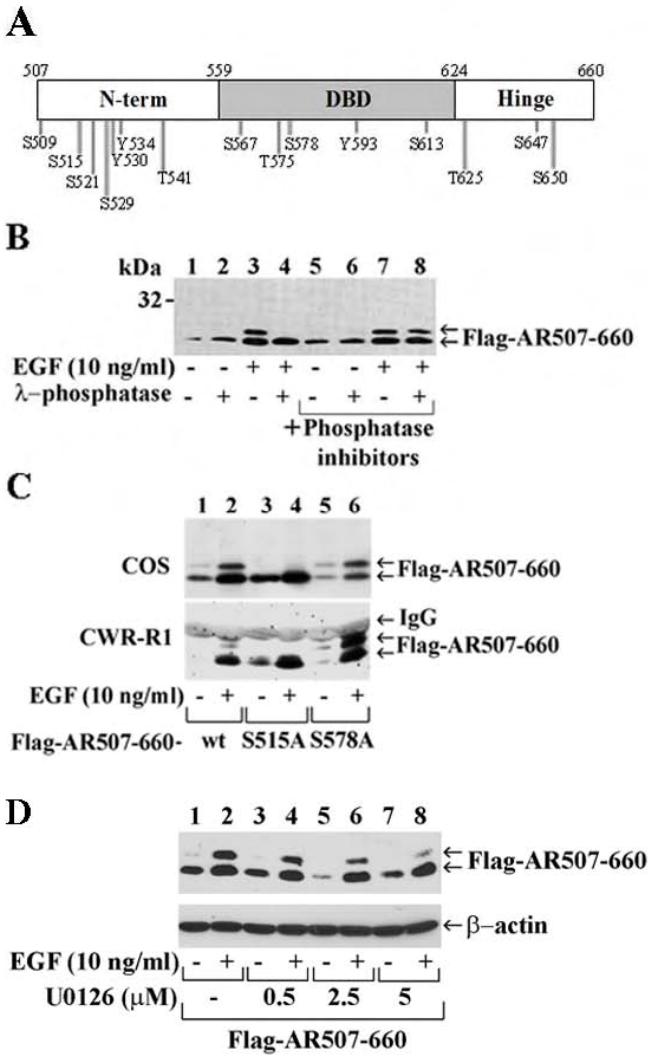

A
507
559
624
660
N-term
DBD
Hinge
S509
S515
S521
S529
Y534
Y530
T541
S567
T575
S578
Y593
S613
T625
S647
S650
B
kDa 1 2 3 4 5 6 7 8
32-
Flag-AR507-660
EGF (10 ng/ml) - - + + - - + +
λ–phosphatase - + - + - + - +
+Phosphatase inhibitors
C
1 2 3 4 5 6
COS
Flag-AR507-660
CWR-R1
IgG
Flag-AR507-660
EGF (10 ng/ml) - + - + - +
Flag-AR507-660- wt S515A S578A
D
1 2 3 4 5 6 7 8
Flag-AR507-660
β–actin
EGF (10 ng/ml) - + - + - + - +
U0126 (μM) - 0.5 2.5 5
Flag-AR507-660

Figure 5

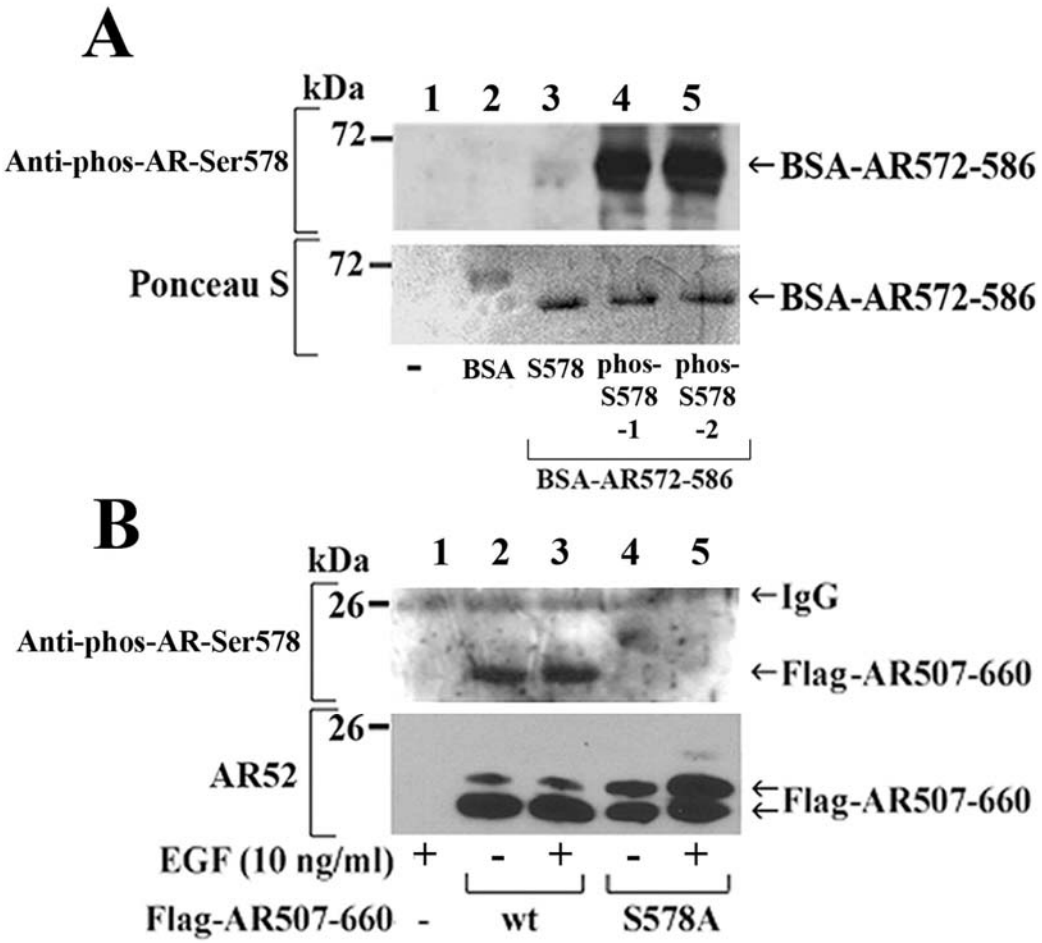

A
kDa
1 2 3 4 5
72
Anti-phos-AR-Ser578
←BSA-AR572-586
72
Ponceau S
←BSA-AR572-586
- BSA S578 phos-S578-1 phos-S578-2
BSA-AR572-586
B
kDa
1 2 3 4 5
26
Anti-phos-AR-Ser578
←IgG
←Flag-AR507-660
26
AR52
←Flag-AR507-660
EGF (10 ng/ml) + - + - +
Flag-AR507-660 - wt S578A

Figure 6

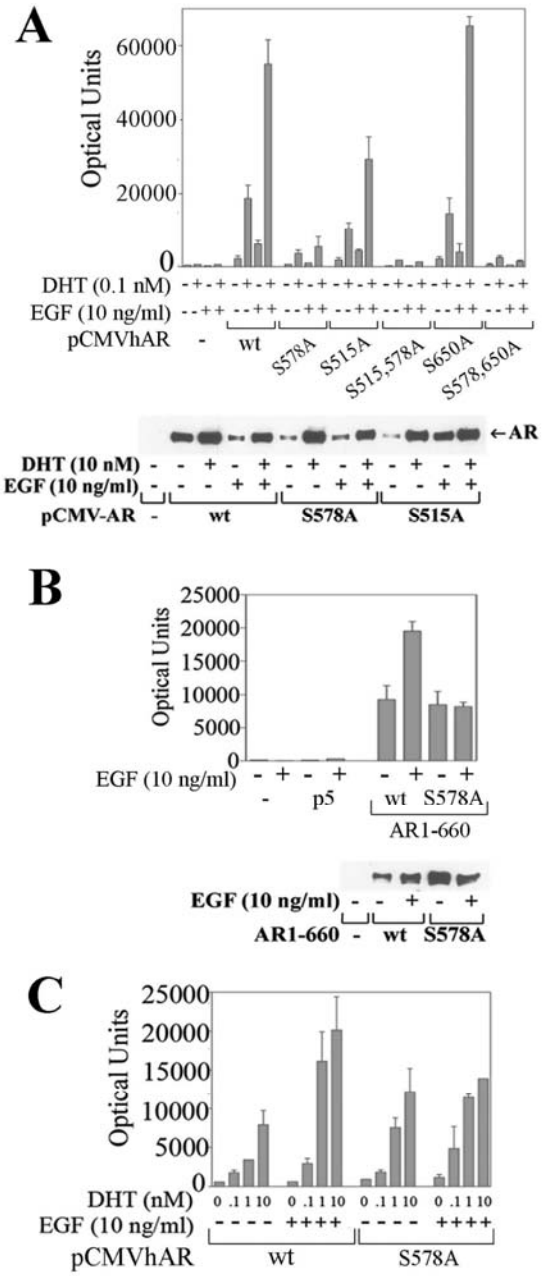

A
Optical Units
60000
40000
20000
0
DHT (0.1 nM)
EGF (10 ng/ml)
pCMVhAR
wt
S578A
S515A
S515,578A
S650A
S578,650A
←AR
DHT (10 nM)
EGF (10 ng/ml)
pCMV-AR
wt
S578A
S515A
B
Optical Units
25000
20000
15000
10000
5000
0
EGF (10 ng/ml)
p5
wt
S578A
AR1-660
EGF (10 ng/ml)
AR1-660
wt
S578A
C
Optical Units
25000
20000
15000
10000
5000
0
DHT (nM)
0 .1 1 10 0 .1 1 10 0 .1 1 10 0 .1 1 10
EGF (10 ng/ml)
- - - - + + + +
pCMVhAR
wt
S578A

Figure 7

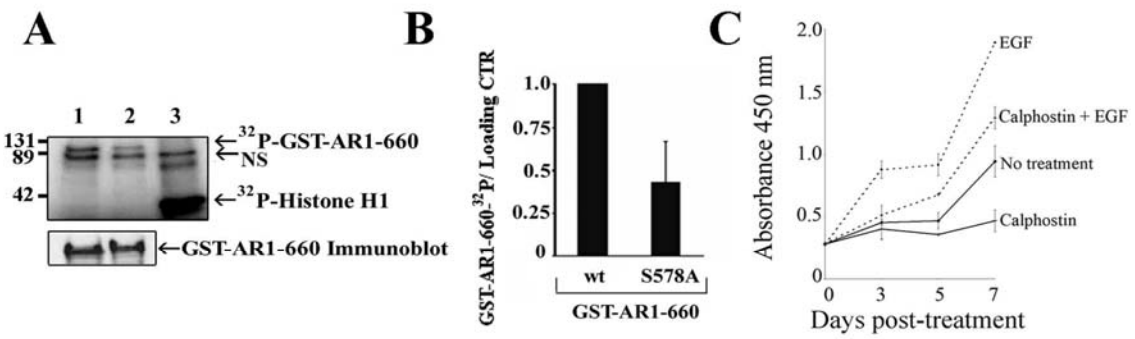

A
B
C
1
2
3
131
89
42
^{32}P-GST-AR1-660
NS
^{32}P-Histone H1
GST-AR1-660 Immunoblot
GST-AR1-660-^{32}P/ Loading CTR
1.0
0.75
0.5
0.25
0
wt
S578A
GST-AR1-660
Absorbance 450 nm
2.0
1.5
1.0
0.5
0
0
3
5
7
Days post-treatment
EGF
Calphostin + EGF
No treatment
Calphostin

Figure 8

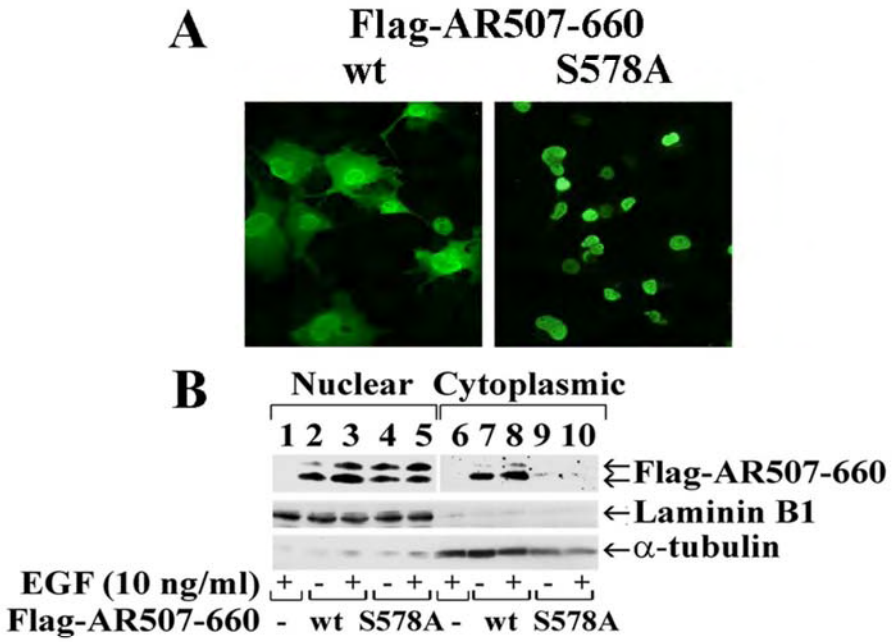
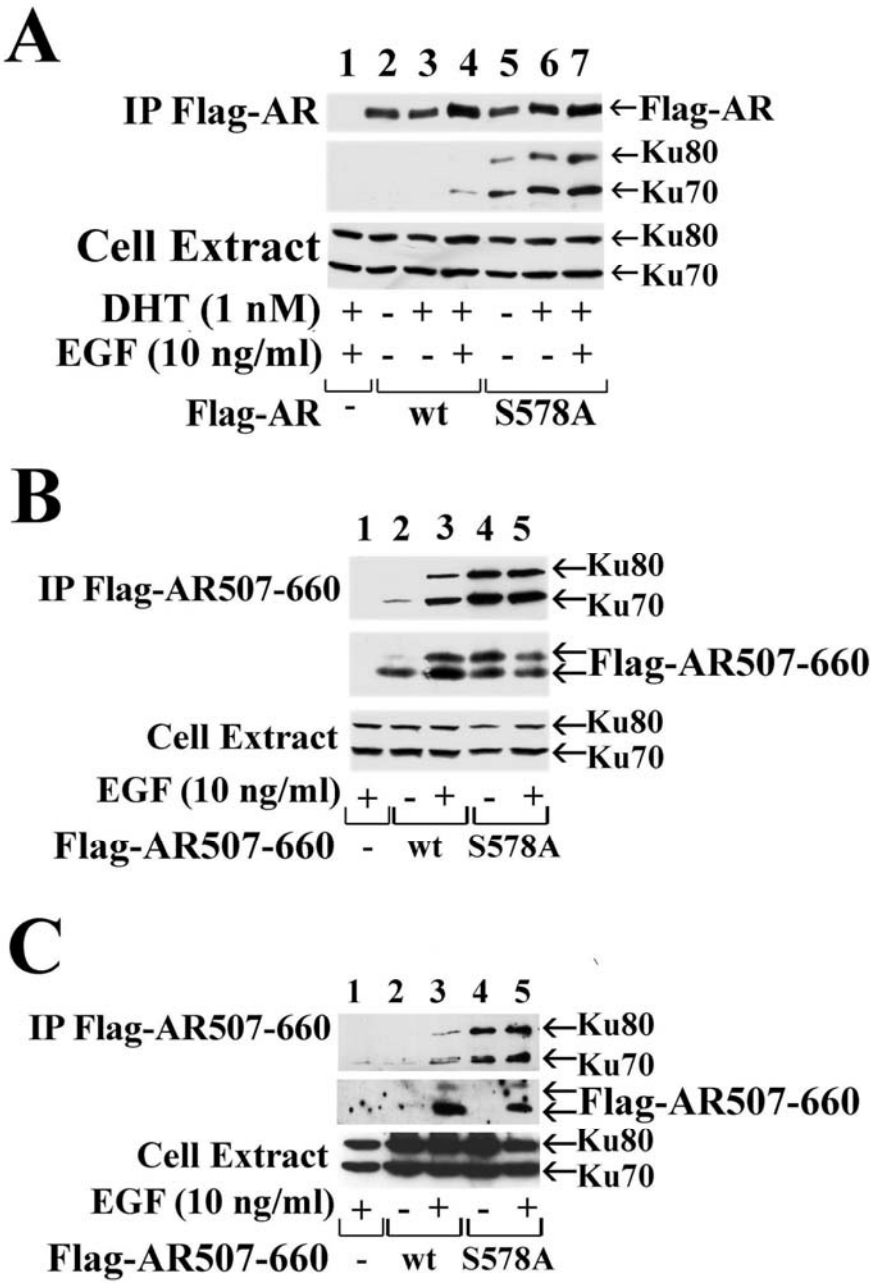

A
Flag-AR507-660
wt
S578A
B
Nuclear
Cytoplasmic
1 2 3 4 5 6 7 8 9 10
Flag-AR507-660
Laminin B1
α-tubulin
EGF (10 ng/ml) + - + - + + - + - +
Flag-AR507-660 - wt S578A - wt S578A

Figure 9


A
1 2 3 4 5 6 7
IP Flag-AR
←Flag-AR
←Ku80
←Ku70
Cell Extract
←Ku80
←Ku70
DHT (1 nM) + - + + - + +
EGF (10 ng/ml) + - - + - - +
Flag-AR - wt S578A
B
1 2 3 4 5
IP Flag-AR507-660
←Ku80
←Ku70
←Flag-AR507-660
Cell Extract
←Ku80
←Ku70
EGF (10 ng/ml) + - + - +
Flag-AR507-660 - wt S578A
C
1 2 3 4 5
IP Flag-AR507-660
←Ku80
←Ku70
←Flag-AR507-660
Cell Extract
←Ku80
←Ku70
EGF (10 ng/ml) + - + - +
Flag-AR507-660 - wt S578A