Regulation of androgen receptor activity by tyrosine phosphorylation

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Summary

The androgen receptor (AR) is essential for the growth of prostate cancer cells. Here, we report that tyrosine phosphorylation of AR is induced by growth factors and elevated in hormone-refractory prostate tumors. Mutation of the major tyrosine phosphorylation site in AR significantly inhibits the growth of prostate cancer cells under androgen-depleted conditions. The Src tyrosine kinase appears to be responsible for phosphorylating AR, and there is a positive correlation of AR tyrosine phosphorylation with Src tyrosine kinase activity in human prostate tumors. Our data collectively suggest that growth factors and their downstream tyrosine kinases, which are elevated during hormone-ablation therapy, can induce tyrosine phosphorylation of AR and such modification may be important for prostate tumor growth under androgen-depleted conditions.

Introduction

Prostate cancer is the second leading cause of cancer death among men in Western countries. Patients with advanced prostate cancer initially benefit from androgen-ablation therapy, which leads to temporary tumor remission due to apoptosis of androgen-sensitive tumor cells. However, recurrence of androgen-independent tumors is inevitable for most patients and renders the conventional hormone therapy ineffective (Denmeade and Isaacs, 2002). It has, therefore, become a focus of intensive study to understand the mechanisms underlying the development of hormone-refractory prostate cancer (Debes and Tindall, 2004; Feldman and Feldman, 2001). The androgen receptor (AR), a member of the steroid hormone receptor family, is primarily responsible for mediating the physiological effects of androgens by binding to specific DNA sequences, known as androgen response elements (AREs), which regulate transcription of androgen-responsive genes (Heinlein and Chang, 2004). Increasingly, clinical findings demonstrate that a majority of androgen-ablation therapy-resistant prostate cancers still express AR and androgen-dependent genes, indicating that the AR-signaling pathway is functional in the absence of androgens or in the presence of low levels of androgens (Culig et al., 2000). Several independent studies also showed that AR is essential for both hormone-sensitive and -refractory prostate cancer (Chen et al., 2004; Zegarra-Moro et al., 2002).

Mutations and amplification of AR, alterations in protein kinases, growth factors, and nuclear receptor coactivators have all been proposed to modulate AR signaling and may, therefore, play key roles in the development of androgen independence of prostate cancer (Feldman and Feldman, 2001; Gelmann, 2002). Mutations in the ligand-binding domain of AR are shown to broaden the ligand-binding profile of the mutant receptor (Veldscholte et al., 1990; Zhao et al., 2000). However, the frequency of AR mutation is generally low and probably only accounts for less than 10% of the cases surveyed (Taplin et al., 2003). Upregulation of the enzymes involved in steroid synthesis in some recurrent prostate tumors and activation of AR via the intracrine mechanism have also been reported (Titus et al., 2005a). However, the tissue androgen levels did not correlate with clinical prognosis (Titus et al., 2005b). Recently, the increased AR expression level was shown to associate with the development

SIGNIFICANCE

Recurrent prostate cancer is resistant to commonly used hormonal therapy and is the major cause of patient death. Study of altered signaling events in hormone-refractory prostate cancer cells will allow us to develop more effective regimens and more reliable prognostic markers. In this study, we show that the androgen receptor can be phosphorylated by tyrosine kinase Src and such modification appears to be important for prostate cancer cell growth under low-androgen conditions. Our results suggest that the level of AR tyrosine phosphorylation may serve as a diagnostic tool to predict patient outcome in response to hormone-ablation therapy and inhibition of tyrosine phosphorylation of AR may be an effective intervention target for hormone-refractory prostate cancer.

of resistance to antiandrogen therapy (Chen et al., 2004). The cross-talk between growth factor and AR-signaling pathways in prostate cancer cells has been well documented. The expression of several peptide growth factors, such as EGF/TGFα, IL-6, and IGF-1, are reported to be elevated during progression to hormone-refractory human prostate cancer (Bartlett et al., 2005; Di Lorenzo et al., 2002; George et al., 2005; Krueckl et al., 2004; Lorenzo et al., 2003). These autocrine/paracrine factors can either induce the androgen-independent activation of AR transcriptional activity or sensitize AR to low concentrations of androgens (Culig et al., 1994; Gregory et al., 2004; Ueda et al., 2002). A substantial body of literature suggests that AR is regulated directly by phosphorylation. Several protein kinases, including the mitogen-activated protein kinase (MAPK), Akt/PKB, cAMP-activated protein kinase A (PKA), and protein kinase C (PKC), have been shown to modulate AR transcriptional activity by phosphorylating serine or threonine residues in AR or its coactivators, such as the transcriptional intermediary factor 2 (TIF2) and the steroid hormone receptor coactivator 1 (SRC1) (Gregory et al., 2004; Lin et al., 2001; Ueda et al., 2002; Yeh et al., 1999). The expression of erbB2/HER-2/neu, a member of the EGF receptor family tyrosine kinases, is increased in a subset of hormone-refractory LAPC4 xenografts (Craft et al., 1999). Forced overexpression of erbB2 in androgen-dependent prostate cancer cells promotes androgen-independent growth (Craft et al., 1999; Yeh et al., 1999). In addition to erbB2, several other non-receptor tyrosine kinases, including Src, FAK, and Etk/BMX, have been implicated in activation of AR transcriptional activity in response to nonsteroid stimuli such as IL-6 and bombesin (Lee et al., 2001, 2004). It is also shown that Src kinase is directly associated with AR upon androgen treatment through a nongenotropic pathway (Kousteni et al., 2001; Migliaccio et al., 2000). However, the mechanisms by which these tyrosine kinases regulate AR transcriptional activity in prostate cancer cells are not well understood yet.

Here, we report that AR is tyrosine phosphorylated in prostate cancer cells in response to growth factors. Our data suggest that growth factors and their downstream tyrosine kinases, which are elevated during hormone-ablation therapy, can induce tyrosine phosphorylation of AR and such modification may contribute to androgen-independent activation of AR or sensitize AR to low levels of hormone. Our findings provide a mechanism by which hormone-refractory prostate cancer cells continue to grow under androgen-depleted conditions.

Results

Elevated tyrosine phosphorylation of AR in hormone-refractory prostate tumor xenografts

We observed that the level of tyrosine phosphorylation is significantly increased in hormone-refractory prostate xenograft tumors compared to their hormone-sensitive counterparts (Figure 1A). Interestingly, the AR protein immunoprecipitated from hormone-refractory tumors could also be recognized by the anti-phosphotyrosine antibody (Figure 1B), suggesting that AR is tyrosine phosphorylated. Moreover, tyrosine phosphorylation of AR was significantly increased in hormone-refractory tumors compared to their hormone-sensitive counterparts (Figure 1B). To find out which tyrosine kinase might be responsible for the increased AR tyrosine phosphorylation, we examined the activity of a panel of tyrosine kinases, including erbB2, FAK, and Src. We only consistently detected the elevated Src kinase activity in all hormone-refractory samples while the protein level of Src remained largely unchanged (Figure 1C).

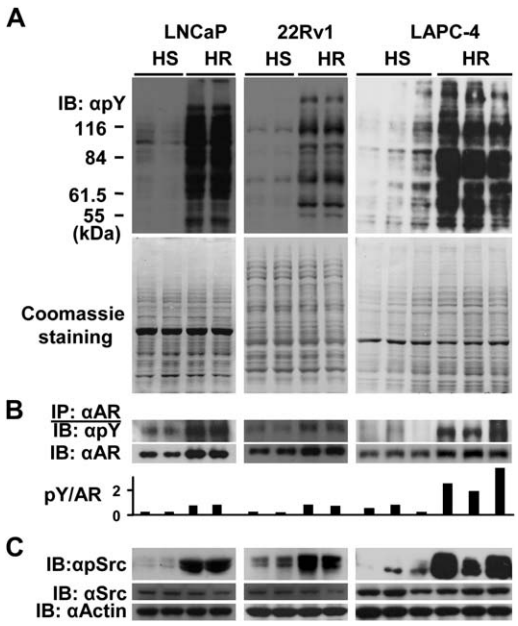

Figure 1. Increased tyrosine phosphorylation in hormone refractory prostate tumor xenografts

A: Hormone-sensitive (HS) and refractory (HR) xenograft tumors were derived as described in Experimental procedures. The tumor lysates were immunoblotted with anti-phosphotyrosine (αpY) antibody. The sample loading was monitored by Coomassie Blue staining (bottom).

B: The immunoprecipitated AR was subjected to immunoblotting with the antibodies indicated. The ratio of pY/AR is shown at the bottom. For each group, HR is higher than HS ($p < 0.05$).

C: Immunoblot with anti-phospho-SrcY416(αpSrc) to determine the Src kinase activity.

Tyrosine phosphorylation of AR in prostate cancer cells

To test whether AR could be tyrosine phosphorylated in prostate cancer cells in response to extracellular stimuli, we treated LNCaP cells with several growth factors and hormones, respectively. As shown in Figure 2A, all tested stimuli, except for DHT, could induce tyrosine phosphorylation of AR in LNCaP cells under our experimental conditions. We also showed that EGF induced a transient tyrosine phosphorylation of AR in all tested AR-positive prostate cancer cell lines with similar kinetics, which peaked at 5 min and tapered by 30 min (Figure 2B). Interestingly, the EGF-induced AR tyrosine phosphorylation was concomitant with the increased phosphorylation of Y416 of Src kinase (Figure 2C), an indicator of Src kinase activity, and was significantly diminished by the selective Src kinase inhibitors PP2 and SU6656 (Figure 2D), as well as the siRNA specific for Src (Figure 2E), suggesting that it is, at least partially, dependent on Src kinase activity. Figure 2F shows that the constitutively active SrcY527F could induce tyrosine phosphorylation of the endogenous AR in LNCaP cells as well as the exogenous AR in COS-1 cells, while the kinase-inactive mutant SrcK295M failed to do so. These data together indicate that EGF-induced AR tyrosine phosphorylation is mediated by Src kinase.

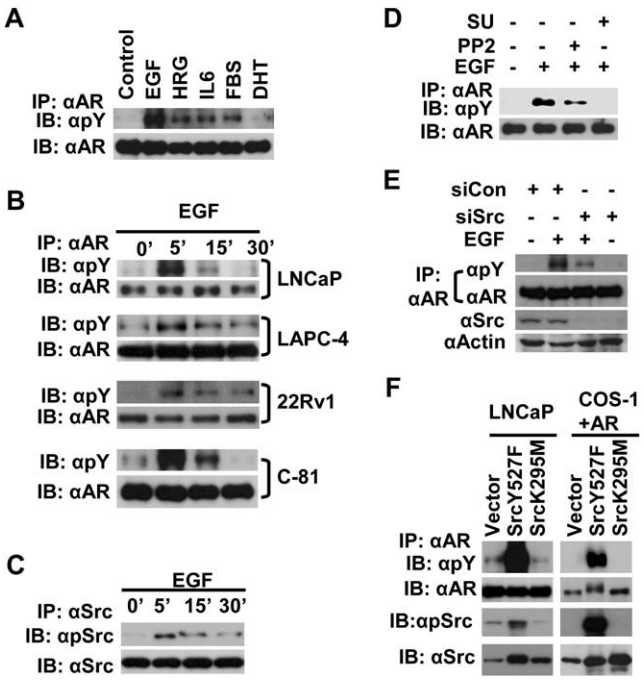

Figure 2. Growth factors induce tyrosine phosphorylation of AR in prostate cancer cells

A: Serum-starved LNCaP cells were treated with 100 ng/ml of EGF, heregulin-β1 (HRG), interleukin-6 (IL-6), 10% (v/v) charcoal-stripped fetal bovine serum (FBS), and 10 nM dihydrotestosterone (DHT) for 5 min. AR tyrosine phosphorylation was determined as described in Figure 1B.

B: Prostate cancer cells were treated with EGF as in (A) for the time indicated. The levels of AR tyrosine phosphorylation were determined as above.

C: Serum-starved LNCaP cells were treated with 100 ng/ml EGF for the time indicated. The cell lysates were immunoprecipitated with anti-Src antibody and followed by immunoblotting with anti-pSrc antibody.

D: LNCaP cells were pretreated with 10 μM PP2 or SU6656 (SU) for 30 min and then treated with 100 ng/ml EGF for 5 min. The AR tyrosine phosphorylation were detected as in (A).

E: LNCaP cells were infected with *lenti*-virus encoding siRNA specific for luciferase (siCon) or for Src (siSrc). After serum-starvation, the infected cells were treated with EGF for 5 min. The AR tyrosine phosphorylation was determined as above. The total cell lysates were immunoblotted with anti-Src and anti-actin antibodies.

F: SrcY527F or SrcK295M were transfected into LNCaP cells or cotransfected with AR into COS-1 cells; the AR tyrosine phosphorylation was detected as in Figure 2A. The total cell lysates were also immunoblotted with anti-pSrc and anti-Src antibodies, respectively.

Identification of AR tyrosine phosphorylation sites using mass spectrometry

Our MS analysis revealed that AR was phosphorylated at multiple tyrosine residues (Figure 3A). All detected tyrosine phosphorylation sites were exclusively present in the sample derived from the cells expressing both AR and the active Src, suggesting that phosphorylation of these sites is induced by Src kinase. We substituted all identified tyrosine residues with a phenylalanine individually to test its effect on Src-induced tyrosine phosphorylation of AR. The mutation of Y534 appears to diminish the Src-induced tyrosine phosphorylation most, suggesting that Y534 is one of the major phosphorylation sites (Figure 3B). Interestingly, the alignment of AR sequences from various species showed that Y534 is evolutionally conserved, suggesting that Y534 may be of functional importance (Figure 3C). To further characterize phosphorylation of AR Y534, we developed a phosphospecific antibody, anti-pARY534. The specificity of this antibody was validated by various assays described in the Supplemental data (Figures S4 and S5). We found that AR Y534 phosphorylation was significantly increased in hormone-refractory tumor xenografts compared to their hormone-sensitive counterparts (Figure 3D). Subsequently, we performed immunohistochemical (IHC) analysis on human prostate tissue arrays by using the antibodies specific for AR, pARY534 and pSrcY416, respectively. As summarized in Table 1 and Figure S7, the phospho-ARY534 staining was detected in all hormone-refractory samples examined. The mean immunostaining scores for pARY534 and pSrcY416 staining in hormone-refractory samples are significantly higher than those in hormone-naive and normal samples ($p < 0.0001$). Although 57.14% of normal and 47.25% of hormone-naive samples in our surveys show weak pSrcY416 staining, only less than 24.14% of both normal and hormone-naive samples are pARY534 positive. It is possible that Src kinase activity may have to reach a certain threshold level in the cells in order to allow pARY534 to become detectable, as phosphorylation of AR is a transient and dynamic event. Our statistical analysis also revealed a positive correlation between the levels of phospho-AR and phospho-Src in the samples examined, suggesting that the increase in AR tyrosine phosphorylation could be, at least in part, due to the elevated Src kinase activity. Figure 3E shows the representative fields of our prostate tissue arrays. The increase in ARY534 phosphorylation in a human hormone-refractory tumor was also confirmed by the Western blot analysis (Figure 3F). Taken together, these data suggest that the increase in AR tyrosine phosphorylation and Src kinase activity is associated with prostate cancer progression.

Tyrosine phosphorylation regulates AR transcriptional activity

Figure 4A shows that EGF induced an approximately 4-fold activation of the ARR2-Luc reporter and this effect was blocked by the pretreatment of cells with the selective Src kinase inhibitor SU6656, suggesting that it is mediated by Src kinase. Figures 4B–4D show that SrcY527F induced the activities of the PSA promoter, the ARR2 promoter, and an artificial promoter composed of five consecutive copies of ARE, suggesting that Src may directly modulate AR-mediated transcriptional activity. Furthermore, EGF-induced AR transcriptional activity could not be blocked by Casodex (Figure 4E), while the DHT-induced AR transcriptional activity was. These data suggest that the effect of EGF is independent of androgen in LNCaP cells. However, the AR siRNA could block both the EGF-induced AR transcriptional activation and PSA production, suggesting that the effect of EGF is mediated by AR (Figure S8).

Figure 4F shows that the substitution of Y534 with phenylalanine significantly inhibited the transcriptional activity of AR induced by EGF or SrcY527F, while the mutant responded to DHT at the concentrations of 1–10 nM as well as the wild-type AR did in COS-1 cells (Figure 4G), suggesting that phosphorylation of Y534 may not be essential for AR activation under normal physiological conditions. However, at low levels of DHT (0.05–0.5 nM), the Y534F mutation significantly diminished the transcriptional activity of AR (Figure 4G). Combination of low doses of androgens and EGF could synergistically enhance the transcriptional activity of ARwt, compared to either one alone (Figure 4H) and such effect was dramatically blocked by ARY534F

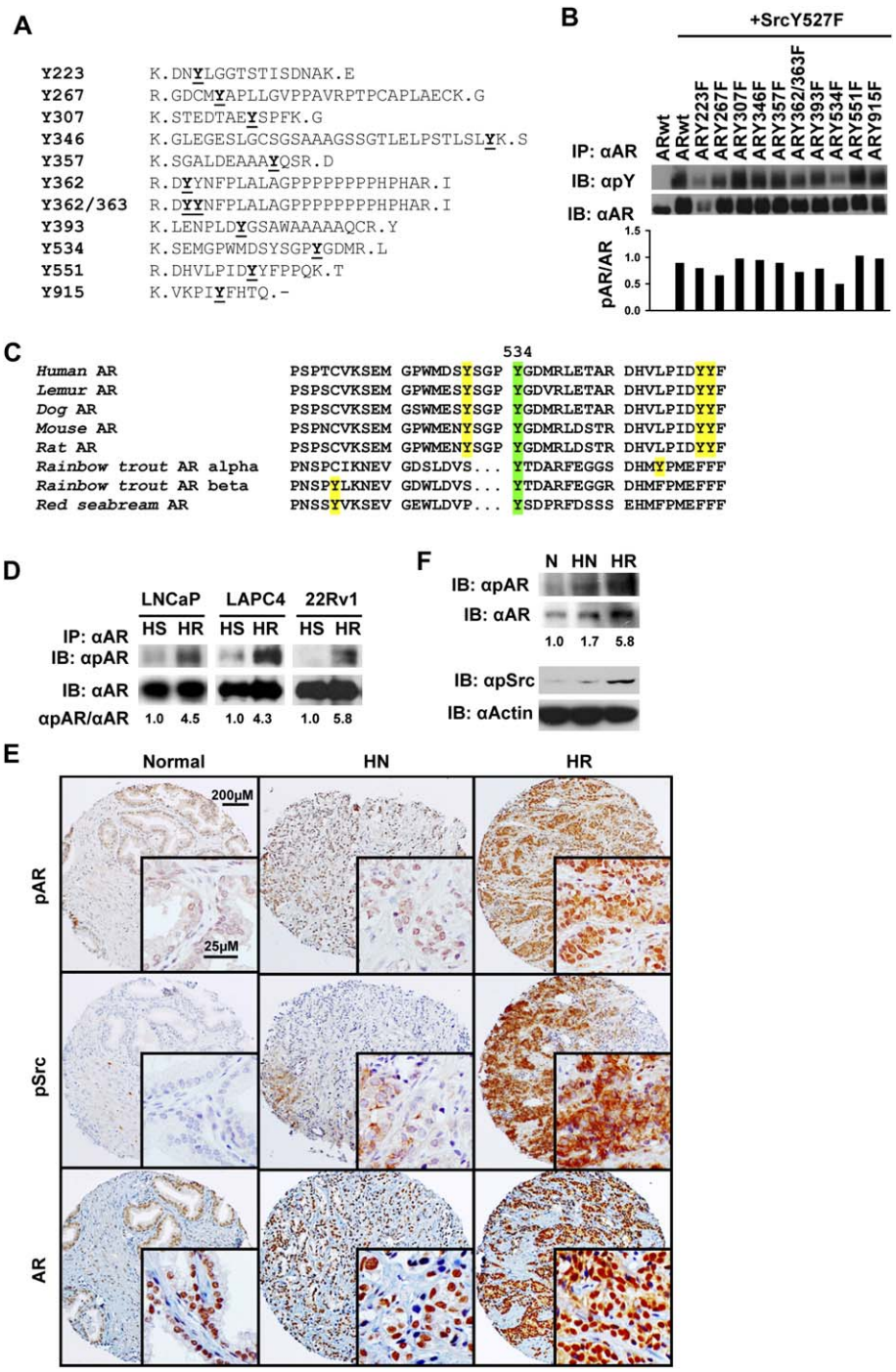

Figure 3. Identification of AR tyrosine phosphorylation sites

A: Mass spectrometry identified AR tyrosine phosphorylation sites (in bold and underlined).

B: COS-1 cells were transfected with the plasmids as indicated. After serum-starvation, the cells were lysed and AR tyrosine phosphorylation was detected as in Figure 1B.

C: Alignment of AR protein sequences to show that Y534 is evolutionarily conserved.

D: Pooled protein extracts from HR and HS tumor xenografts (Figure 1) were immunoprecipitated with anti-AR antibody, followed by immunoblotting with anti-pARY534 (αpAR) antibody. The fold changes of the pAR/AR ratio compared to HS sample were shown (bottom).

E: Representative fields of normal prostate tissue, hormone-naive (HN), and hormone-refractory (HR) human prostate tumor arrays immunohistochemically stained with anti-pAR, anti-pSrc, and anti-AR antibodies as indicated.

F: The lysates of a HR tumor, the pooled HN tumors, and the pooled normal prostate tissues were immunoblotted with the indicated antibodies. The fold change in AR tyrosine phosphorylation was labeled as in (D).

mutation. Taken together, these data suggest that phosphorylation of Y534 is required for the growth factor-induced ligand-independent activation of AR, as well as for the optimal activation of AR induced by low levels of androgen.

Consistent with the reporter assays, we found that both EGF and SrcY527F could induce an increase in PSA protein level, which could be blocked by SU6656 (Figure S9). These data further confirmed the effects of EGF and Src kinase on AR transcriptional activation. To test whether phosphorylation of Y534 is required for PSA expression, we replaced the endogenous AR with the mutant AR carrying Y534F mutation by using the "AR replacement" strategy described in Figure 4I, which allowed us to assess the contribution of a specific mutation in AR to its biological activity under the conditions close to the natural milieu. The replacement of the endogenous AR in LNCaP cells was achieved by knocking down the endogenous AR via the siRNA specific for AR and simultaneously introducing back an exogenous codon-switched AR (ARcs) cDNA that contains three silent mutations in the siRNA target sequence. Introduction of Y534F mutation on the ARcs backbone allowed us to express the mutant ARcs-Y534F in cells expressing the siRNA targeting the endogenous AR. The replacement of AR was carried

Table 1. Association of phosphorylation of AR and Src with prostate cancer progression

	pARY534					
	Nucleus		Cytoplasm		pSrcY416	
Group	Main ± SE	N (+%)	Mean ± SE	N (+%)	Mean ± SE	N (+%)
Normal	0.6449 ± 0.2822	46(10.87)	0.1033 ± 0.0723	46(4.35)	1.5691 ± 0.2708	35(57.14)
HN	1.2893 ± 0.2647	87(24.14)	1.1303 ± 0.2422	87(23.00)	1.6960 ± 0.2265	91(47.25)
HR	5.8190 ± 0.4051	18(100)	6.0278 ± 0.2548	18(100)	4.9352 ± 0.5019	18(94.44)
p Value	<0.0001		<0.0001		<0.0001	

The immunostaining scores (mean ± standard error [SE]) for pARY534 (both in the cytoplasm and nucleus) and pSrcY416 in normal epithelium, hormone-naive (HN), and refractory (HR) prostate tumor foci are summarized. N represents the number of the cases with the percentage of the positive staining cases (+%) in the parentheses. A total of 151 cases were analyzed for pARY534 staining and 144 cases for pSrcY416 staining. The statistical differences between HR and HN in immunoreactivity with each antibody were determined by Wilcoxon rank sum test. The immunoreactive scores of HR are significantly higher than those of HN tumors (*$p < 0.0001$). The Pearson correlation coefficient ρ of the immunoreactive scores between total pAR (cytoplasm plus nucleus) and pSrc is 0.32538 ($p < 0.0001$).

out by coinfection of the lenti-viruses encoding the ARcs and the siRNA specific for AR at >95% efficiency in all prostate cancer cells tested. We replaced the endogenous AR with the ARcs-wt or ARcs-Y534F in LNCaP cells and then examined the effects of Y534F mutation on the PSA production induced by DHT and EGF. Figure 4J shows that the PSA production of the cells expressing the ARcs-Y534F mutant was significantly reduced in comparison to that of the cells expressing the ARcs-wt in response to EGF or to low levels (0.01 and 0.1 nM) but not high levels (1 and 10 nM) of DHT. Taken together, these results suggest that Y534 phosphorylation of AR is required for PSA production in prostate cancer cells stimulated by low levels of DHT or by growth factors under the androgen-depleted conditions.

Tyrosine phosphorylation regulates AR translocation

Translocation of AR from the cytoplasm into the nucleus is required for AR to exert its transcriptional activity in cells, we therefore examined whether phosphorylation of Y534 could influence AR nuclear translocation in prostate cancer cells. Immunofluorescence microscopy revealed that SrcY527F could induce the nuclear translocation of AR in LNCaP cells in the absence of androgens (Figure 5A). The SrcY527F-promoted nuclear translocation of AR was also observed in PC-3 cells expressing the exogenous wild-type AR. As shown in Figure 5B, the nuclear translocation of AR was only detected in the cells expressing the active Src (arrows), while AR was primarily in the cytosol of the cells lacking the active Src (arrowheads). However, the majority of the ARY534F mutant remained in the cytosol and failed to translocate into the nucleus even in the presence of the active Src. Cell fractionation also showed that SrcY527F could significantly increase the level of ARwt but not ARY534F in the nuclear fraction (Figure 5C). As a positive control, 10 nM DHT dramatically increased the levels of AR in the nuclear fraction, regardless of Y534 mutation. These data suggest that phosphorylation of Y534 may be required for the Src-promoted nuclear translocation of AR under androgen-depleted conditions.

AR tyrosine phosphorylation regulates prostate cancer cell growth under androgen-depleted conditions

Figure 6A shows that growth of C-81 cells in androgen-depleted medium was significantly increased by EGF, and this effect can be blocked by Src siRNA or AR siRNA. This suggests that EGF is driving AR-dependent cell growth under androgen-depleted condition and Src kinase is required for this process. Moreover, the Src siRNA dramatically inhibited the growth of C-81 and CWR-R1 cells in castrated male nude mice (Figures 6B and 6C), and such inhibition coincided with diminished ARY534 phosphorylation, while phosphorylation of MAPK remained largely unchanged (Figure 6D), implying that Src kinase may be required for the growth of hormone-refractory prostate cancer cells in castrated mice possibly by phosphorylating AR.

In addition, we replaced the endogenous AR with the ARcs-wt or ARcs-Y534F in an AR-positive androgen-independent prostate cancer cell line, CWR-R1, as described in Figure 4I. The effects of Y534F mutation on cell proliferation in the androgen-depleted medium were examined. Similar to what we observed in C-81 cells (Figure 6A), EGF can increase the growth of CWR-R1 cells, and this effect can be inhibited by the siRNA for either AR or Src (Figure 7A). Moreover, reintroducing of the exogenous wild-type AR(ARcs-wt) back to CWR-R1 cells could partially rescue the growth inhibition caused by the AR siRNA, while the ARcs-Y534F mutant failed to do so. Furthermore, in castrated male mice, growth of LNCaP tumor xenografts expressing SrcY527F is significantly increased compared to the vector control (Figure 7B), and such effect was significantly blocked when the endogenous AR was replaced with ARcs-Y534F (Figure 7C), suggesting that the active Src can promote prostate cancer cell growth under androgen-depleted conditions and it is dependent on ARY534 phosphorylation. To further examine whether phosphorylation of Y534 is required for the growth of hormone-refractory prostate cancer cells, the endogenous AR in hormone-resistant CWR-R1 cells was replaced with the ARcs-wt or ARcs-Y534F. The growth of the cells was examined in the castrated male nude mice. Figure 7E shows that the sizes of the tumors expressing the ARcs-Y534F mutant were significantly smaller than those of the tumors expressing the ARcs-wt, suggesting that the integrity of Y534 is important for the growth of CWR-R1 in the castrated male nude mice. In contrast, these cells grew equally well in the intact male mice, regardless of Y534 mutation (Figure 7F). Taken together, our results demonstrated that Src-induced ARY534 phosphorylation is important for prostate tumor growth under androgen-depleted conditions.

Tyrosine phosphorylation is required for the recruitment of AR to the chromatins

Because AR regulates gene expression primarily through binding to AREs in the promoter/enhancer region of its target genes, we examined whether tyrosine phosphorylation of AR could

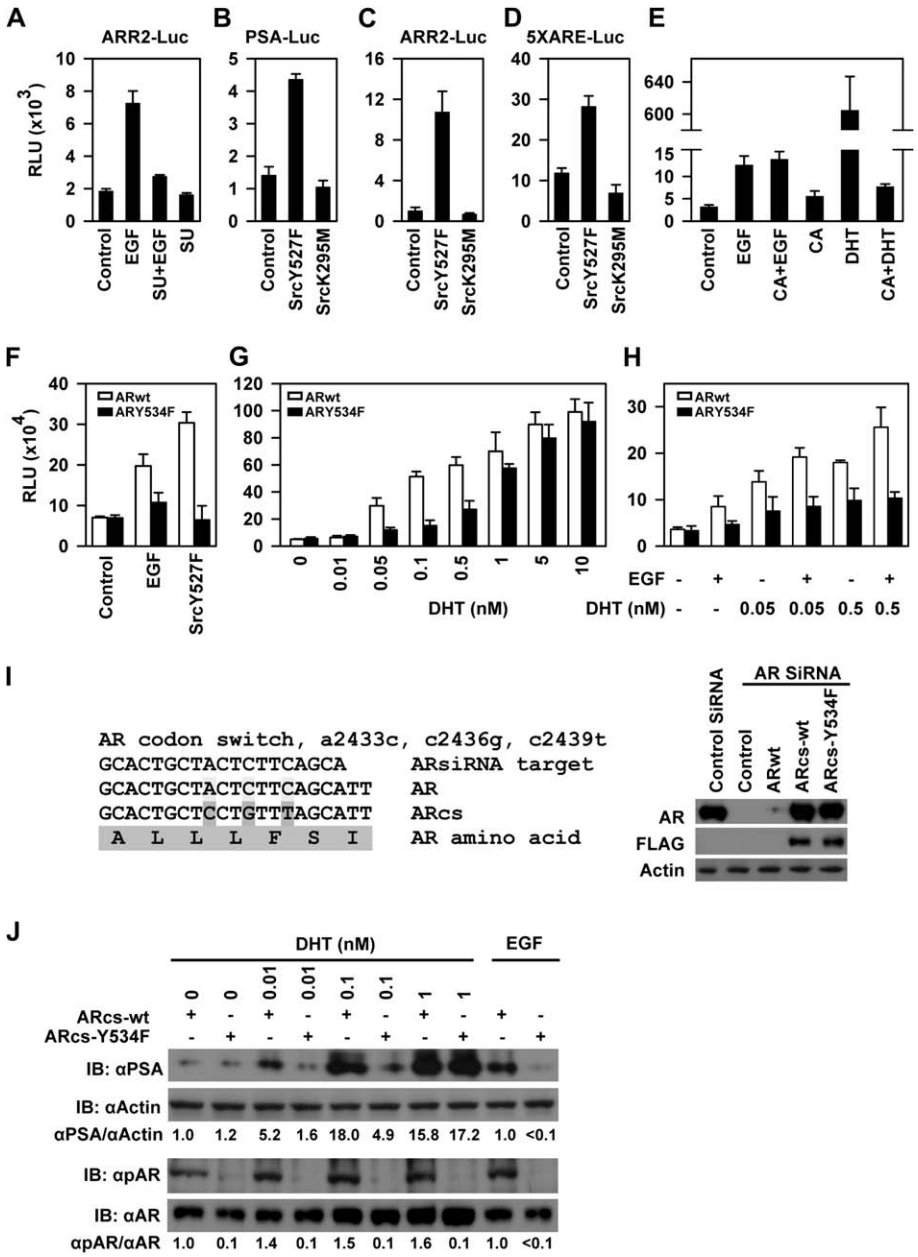

Figure 4. Modulation of AR transcriptional activity by tyrosine phosphorylation

A: LNCaP cells were transfected with ARR2-Luc reporter. After serum-starvation, the cells were pretreated with or without 2 μM SU6656 (SU) for 30 min then treated with 50 ng/ml EGF for 16 hr before the luciferase activity was measured. The results were presented as the mean relative light units (RLU) ± SD of the triplicate samples.

B–D: LNCaP cells were transfected with PSA-Luc (B), ARR2-Luc (C), or 5xARE-Luc (D), along with the vector control, SrcY527F or SrcK295M, as indicated after serum-starvation overnight, the luciferase activity was determined as in (A).

E: LNCaP cells were transfected with ARR2-Luc. After serum-starvation, the cells were pretreated with 10 μM Casodex (CA) for 30 min then treated with 50 ng/ml EGF or 1 nM DHT for 16 hr before the luciferase activity was determined as in (A).

F: COS-1 cells were transfected with ARR2-Luc along with ARwt or ARY534F; the EGF or SrcY527F-induced luciferase activity was determined.

G: COS-1 cells cultured in the medium with charcoal-stripped serum were transfected with ARwt or ARY534F along with the ARR2-Luc and then treated with DHT at the indicated doses for 24 hr. The luciferase activity was determined as in (A).

H: COS-1 cells were transfected with ARwt or ARY534F, along with the ARR2-Luc. After serum-starvation, the cells were treated with 50 ng/ml EGF and DHT at different doses as indicated. The luciferase activity was determined.

I: AR-replacement strategy. LNCaP cells were coinfected with the *lenti*-virus encoding AR siRNA, and the FLAG-tagged wild-type AR (ARwt), FLAG-tagged codon-switched wild-type AR (ARcs-wt), or FLAG-tagged codon-switched Y534F mutant (ARcs-Y534F). At 48 hr postinfection, the AR proteins were examined by immunoblotting with anti-AR or anti-FLAG antibodies as indicated (right panel).

J: Following AR replacement, the infected LNCaP cells were incubated in the medium containing 5% charcoal-stripped serum with different doses of DHT or incubated in the serum-free medium with 10 ng/ml EGF for 2 days. The levels of PSA and pAR from the immunoblots were normalized by calculating the ratios of PSA/actin and pAR/AR, respectively. The changes in fold compared to the control (0 nM DHT or EGF-treated ARwt) were shown (bottom).

modulate its recruitment to those of *PSA* and *KLK2* by using a chromatin immunoprecipitation (ChIP) assay. We first tested whether EGF could induce AR recruitment to the promoter/enhancer region of *PSA* and *KLK2* in LNCaP cells with the DHT treatment as a positive control. Figure 7G shows that DHT could induce the AR occupancy of the ARE sites located in both the promoter and the enhancer regions of *PSA* and *KLK2* genes as reported previously (Shang et al., 2002). However, EGF treatment only promoted AR binding to the promoter ARE site(s) but not the enhancer ARE site(s) of these genes, suggesting that the differential recruitments of AR to distinct AREs could occur in response to different stimuli. Pretreatment of LNCaP cells with SU6656 completely abolished the EGF-induced AR occupancy on the promoter sites. Using the AR-replacement strategy, we also found that EGF failed to induce the recruitment of the ARY534F mutant to the promoter sites of both genes (Figure 7H). Taken together, these results suggest that Src-induced Y534 phosphorylation is required for EGF-induced recruitment of AR to the ARE-containing promoter/enhancers. In addition, the EGF-induced transcription of endogenous AR target genes, including *PSA*, *KLK-2*, and *POV-1*, was inhibited by Y534F mutation as determined by quantitative real-time RT-PCR (Figure S10). This further supports our hypothesis that growth factor-induced AR tyrosine phosphorylation may regulate AR target gene transcription under androgen-depleted conditions.

Discussion

Growth factors have long been proposed to play a role in the regulation of AR transcriptional activity, especially under the androgen-depleted conditions. However, the mechanisms by

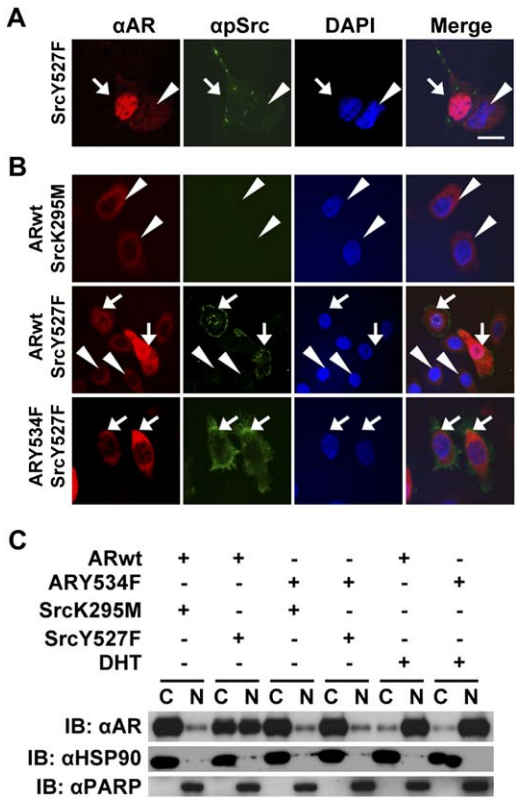

Figure 5. Regulation of AR nuclear translocation by tyrosine phosphorylation

A: LNCaP cells were transfected with SrcY527F. After serum-starvation, immunofluorescence confocal microscopy was carried out by costaining cells with anti-AR (red) and anti-pSrc (green) antibodies as described (scale bar, 5 μm). Arrow: SrcY527F-positive cell; arrowhead: SrcY527F-negative cell.

B: PC-3 cells were first infected with *lenti*-virus encoding ARwt or ARY534F. The infected cells were transiently transfected with SrcY527F or SrcK295M and stained as in (A).

C: The infected and transfected PC-3 cells from (B) were serum-starved overnight and treated with or without 10 nM DHT for 1 hr. The cell fractionation was carried out as described in Supplemental data. Equal amounts of cytoplasmic (C) and nuclear (N) fraction were immunoblotted. HSP90 and PARP were used as markers of the cytoplasmic and nuclear fractions, respectively.

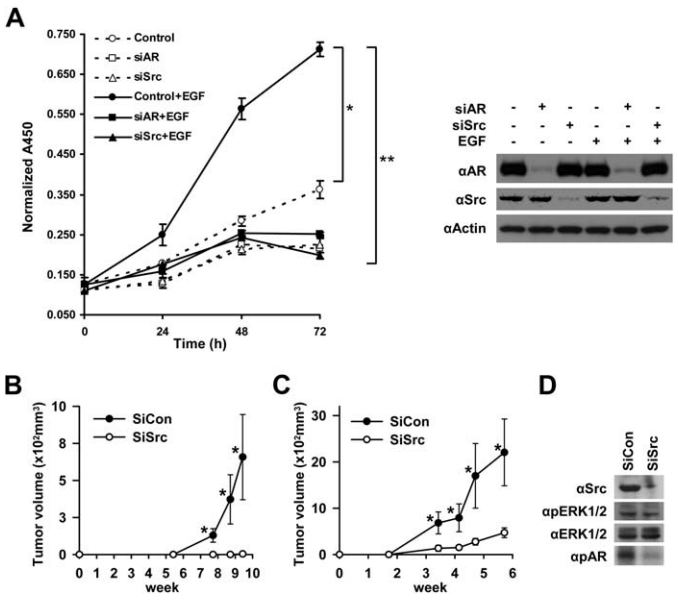

Figure 6. Src kinase inhibition leads to growth attenuation under androgen-depleted conditions

A: C-81 cells were infected with the *lenti*-virus encoding the siRNA specific for luciferase (control), AR (siAR), or Src (siSrc). The cell growth was monitored using WST-1 assay. *Control + EGF versus Control at 48 hr and 72 hr ($p < 0.01$); **Control + EGF versus siAR + EGF/siSrc + EGF at 48 and 72 hr ($p < 0.01$). The data were expressed as the average of normalized triplet samples (mean ± SD). The levels of AR and Src in these cells were detected by immunoblotting at 48 hr postinfection (right panel).

B–D: C-81 (B) and CWR-R1 (C) cells were infected with the *lenti*-virus encoding the siRNA specific for luciferase (SiCon) or Src (SiSrc). At 48 hr postinfection, the cells were injected into the left and right flank of the castrated male nude mice, respectively, and growth of the tumors were examined as described. *$p < 0.01$. The result represents the mean tumor volume ± SE (n = 5 mice/group). Immunoblots of Src, phospho-ERK1/2 (pERK1/2), ERK1/2, and pAR in the CWR-R1 tumors (D).

which nonsteroid peptide growth factors modulate AR activity are not well understood, though several studies have shown that it may be mediated by direct phosphorylation of AR or its cofactors through a kinase cascade involving some serine/threonine kinases, as reported previously. We have observed that the overall level of tyrosine phosphorylation is significantly increased in hormone-refractory human prostate tumor xenografts derived from castrated male mice, suggesting that tumor cells may utilize the autocrine/paracrine factors activating tyrosine kinases to compensate for loss of androgens. Furthermore, we have provided biochemical evidence that AR is tyrosine phosphorylated in response to several growth factors, including EGF, heregulin, and IL-6. These factors are known to be either highly expressed in prostate tissues or elevated in prostate tumors. EGF is one of most abundant growth factors present in prostate tissues and promotes the survival and growth of prostate epithelial cells. IL-6 is negatively regulated by androgens and upregulated upon castration in murine models (Bellido et al., 1995). Several independent studies showed that IL-6 can also activate AR transcriptional activity in the absence of androgens and promote androgen-independent growth of human prostate cancer cells in mouse xenograft models (Lee et al., 2003b; Steiner et al., 2003). Therefore, these factors and their downstream tyrosine kinases may account for, at least in part, the increased tyrosine phosphorylation level of AR and other proteins in hormone-refractory tumor xenografts.

Several independent attempts were made to identify the phosphorylation sites of AR, and only phosphoserines or phosphothreonines were uncovered in these studies (Gioeli et al., 2002; Zhou et al., 1995). The nature of rapid and transient tyrosine phosphorylation of AR induced by growth factors may make it difficult to be captured by the conventional metabolic labeling methods and therefore may explain why tyrosine phosphorylation of AR was not detected by the previous studies. We have identified multiple tyrosine phosphorylation sites in AR induced by the Src kinase in vivo by the MS analysis. Substitution of Y534, one of the major phosphorylation sites induced by Src kinase, with phenylalanine has profound effects on AR-mediated transcription and growth. Our findings suggest that Src-induced phosphorylation of Y534 in AR may play an important role in the survival/growth of prostate cancer cells under androgen-depleted conditions. At this time, we still do not know whether phosphorylation of ARY534 alone is sufficient to confer

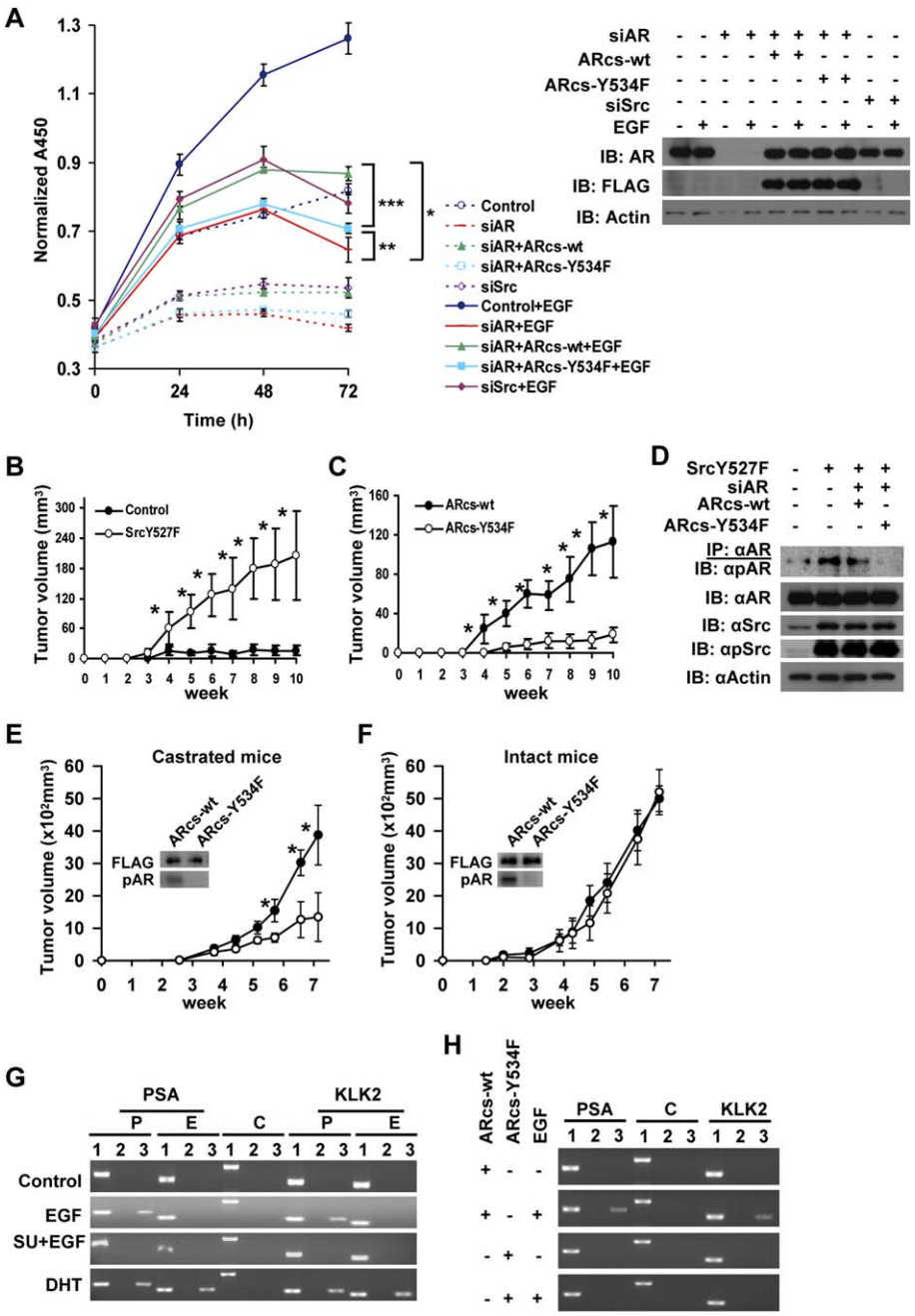

Figure 7. Effect of AR tyrosine phosphorylation on prostate cancer cell growth under androgen-depleted conditions

A: CWR-R1 cells were infected with the *lenti*-virus encoding AR siRNA (siAR), Src siRNA (siSrc), ARcs-wt, and ARcs-Y534F as indicated. The cell growth was monitored using WST-1 assay. *siAR + ARcs-wt + EGF versus siAR + EGF at 48 and 72 hr ($p < 0.01$); **siAR + ARcs-Y534F + EGF versus siAR + EGF at 48 and 72 hr ($p > 0.05$); ***siAR + ARcs-wt + EGF versus siAR + ARcs-Y534F + EGF at 48 hr and 72 hr ($p < 0.05$). The data were expressed as the average of normalized triplet samples (mean ± SD). The levels of AR in these cells were detected by immunoblotting at 48 hr postinfection as in Figure 4I (right panel).

B–D: LNCaP cells were infected with *lenti*-virus encoding SrcY527F and vector control (B); the AR in LNCaP cells were replaced with ARcs-wt or ARcs-Y534F and coinfected with *lenti*-virus encoding SrcY527F(C). At 48 hr postinfection, the cells were injected into castrated male SCID mice. The result represents the mean tumor volume ± SE (n = 5 mice/group). *$p < 0.05$. The levels of pAR, AR, pSrc, and Src in the cells were detected by immunoprecipitation and immunoblotting at 48 hr postinfection (D).

E and F: After AR replacement, the CWR-R1 cells expressing ARcs-wt (solid) or ARcs-Y534F (open) were injected into the castrated (E) and intact (F) male nude mice. The result represents the mean tumor volume ± SE (n = 5 mice/group). *$p < 0.01$. Inset, Western blots of FLAG-AR and pAR of the CWR-R1 xenograft tumors.

G and H: LNCaP cells were treated for 1 hr with 100 ng/ml EGF, 10 nM DHT, or pretreated with SU6656 (SU) for 30 min and followed by EGF treatment (SU + EGF). The binding of AR to the promoter (P) or the enhancer (E) of *PSA* or of *KLK2* or to an irrelevant control region (C) was analyzed by ChIP assay using anti-AR antibodies (G). Following AR replacement, the LNCaP cells were treated with 100 ng/ml EGF for 1 hr, and the AR binding to the promoter of *PSA* or of *KLK2* and an irrelevant control region (C) was analyzed by ChIP using anti-FLAG antibodies (H). PCR products from input (1), immunoprecipitation with control antibody (2), and with anti-AR/FLAG antibody (3) were resolved on agarose gels.

androgen-independence, as the ARY534D/E mutants failed to promote prostate cancer cell growth under androgen-depleted conditions (Z.G. and Y.Q., unpublished data). One explanation could be that the D/E mutation may not faithfully mimic the tyrosine phosphorylation of AR. Given that AR is phosphorylated at multiple tyrosine residues, it is also possible that multiple phosphorylation events may act synergistically and are required for Src-induced activation of AR. Currently, we are actively investigating the functional significance of other identified tyrosine phosphorylation sites and their possible contribution to hormone-independent growth of prostate cancer cells.

In this report, we showed that non-receptor tyrosine kinase Src activity is elevated in hormone-refractory prostate tumors. Although Src was identified as an oncogene in animal models several decades ago, the evidence of mutation or overexpression of Src family kinases in human prostate cancer is scant, while it has been shown that Src kinase is overexpressed in breast and colon cancer patients (Biscardi et al., 2000; Yeatman, 2004). Given that Src kinases can be activated by a variety of extracellular stimuli, including growth factors and matrix proteins, it is likely that upregulation of its kinase activity by the deregulated autocrine/paracrine factors or microenviroment may be a more common mechanism by which Src kinases contribute to human malignancies. It is possible that some other tyrosine kinases activated by Src, such as FAK or Etk/BMX, may also be involved in the induction of AR tyrosine phosphorylation in a subset of tumors where these kinases are overexpressed due to the heterogeneous nature of prostate cancers. This is

supported by our observation that the level of phospho-AR is also elevated in the Etk transgenic mouse prostate (B.D. and Y.Q., unpublished data). In addition, overexpression of erbB2 has been reported in a subset of hormone refractory tumors. However, under our experimental conditions, the erbB2 inhibitor AG879 has little effect on EGF-induced ARY534 phosphorylation, suggesting that erbB2 may exert its effect via a distinct pathway (e.g., MAPK pathway), as suggested by previous studies, other than phosphorylating ARY534.

It is still unclear how tyrosine phosphorylation modulates AR transcriptional activity. One possible scenario is that tyrosine phosphorylation of AR may induce conformational change of AR, which mimics the ligand-binding effect in the absence of androgens or facilitates the ligand binding in the presence of extremely low level of androgens. This is corroborated by our observation that Y534 mutation diminishes the Src-promoted nuclear translocation of AR. Phosphorylation may also act as a conformation switch to control the transition of AR from the inactive to the active conformation, which allows AR to dissociate from the negatively regulatory proteins (e.g., HSP90) upon being tyrosine phosphorylated and subsequently dimerize and/or associate with the nuclear import machinery complex in the absence of androgens. Future studies to identify the associated proteins with AR in the active conformation will allow us to understand the mechanisms by which Src promotes AR nuclear translocation.

One issue for debate on the growth factor-induced androgen-independent activation of AR transcriptional activity is that growth factor (e.g., EGF or IL-6) alone in general can only induce a few-fold increase of AR transcriptional activity in the serum-free medium, which seems to be negligible in comparison to the magnitude of the optimal induction by androgens. However, based on the studies of the dose response of LNCaP cells to androgen in tissue culture (Berns et al., 1986; Olea et al., 1990), low levels of androgens (0.01–0.1 nM) promote while high levels of androgens (1–100 nM) inhibit LNCaP cell proliferation. The induction of AR transcriptional activity in tissue culture by low levels of androgens is also in the range of a few-fold increase, which is comparable to that induced by growth factors. Thus, it is possible that a few-fold increase of AR transcriptional activity may be sufficient for regulating a subset of AR-regulated genes that are required for promoting the survival/growth of prostate cancer cells. In addition, the differential recruitment of AR to the AREs present in the different chromatin context by growth factors may preferentially turn on a subset of AR-regulated survival/growth-promoting genes. Therefore, it is possible that autocrine/paracrine loops, which generate high local concentrations of various growth factors, may be sufficient to maintain AR transcriptional activity and promote the survival and growth of these cells under the androgen-depleted conditions.

On the other hand, at this time, we cannot exclude the possibility of the presence of an extremely low level of androgens or some androgen-like factors in the reagents used in our experiments. As a matter of fact, no absolutely androgen-free circumstance exists under physiological/pathological conditions, since the level of residual androgens in patients treated with the ablation therapy might not be as low as we previously thought. In spite of that, DHT alone does not induce appreciable tyrosine phosphorylation of AR in LNCaP cells in the serum-free medium; a low level of AR tyrosine phosphorylation is detectable in normal mouse prostate tissues (Z.G. and Y.Q., unpublished data) as well as in human benign prostate tissue samples. Thus, tyrosine phosphorylation of AR may also play a role in modulation of AR activity in the normal prostate gland, though such modulation may not be essential for AR-mediated transcription regulated by androgens at normal physiological levels as suggested by our data. However, upon androgen ablation, the dependency on ARY534 for its transcriptional activity may become pronounced. This is supported by our observations that both integrity of ARY534 and Src kinase activity are required for AR-mediated transcriptional activity at low levels of androgens and prostate cancer cell growth in castrated mice, and AR tyrosine phosphorylation is significantly increased in hormone-refractory tumors, as detected by our anti-pARY534 antibody. These findings may be of clinical importance, as hormone-refractory tumor cells appear to utilize the autocrine/paracrine factors activating tyrosine kinases to promote AR transcriptional activity after the androgen-ablation therapy. Therefore, the combinatory therapy with the inhibitors targeting at the tyrosine kinases (e.g., Src and Etk), whose activities are elevated after the ablation therapy, may be a more effective regimen for advanced prostate cancer.

In this study, we showed that tyrosine phosphorylation of AR is elevated in human hormone-refractory prostate cancer. It is noteworthy that over 65% (13 out of 20) of the pARY534-positive hormone-naive samples are of Gleason sum greater than 8. Previous studies suggested that patients having poorly differentiated tumors with high Gleason sum are likely resistant to hormonal therapy in nature, and approximately 15% of prostate cancer patients will not respond to hormone manipulation. It has yet to be determined whether the pARY534 antibody could be used for identifying these patients, at least in part, prior to hormonal therapy. If so, the status of AR tyrosine phosphorylation may be used as a parameter to predict the patient outcomes in response to androgen-ablation therapy in the future.

Experimental procedures

Phosphorylation site mapping by mass spectrometry

To map the phosphorylation sites of AR induced by Src, COS-1 cells were cotransfected with expression vectors encoding AR and SrcY527F. AR protein was immunoprecipitated and then purified by SDS-PAGE. The AR protein was visualized by Coomassie Blue staining and excised. The gel slice was subjected to digestion with trypsin (Promega) overnight at 37°C. The peptides were extracted and analyzed as described previously (Yu et al., 2004). The raw MS/MS data were searched by using SEQUEST (ThermoFinnigan, San Jose, CA) against a database consisting of the androgen receptor protein sequence. Phosphorylation sites were determined by allowing a dynamic modification of 79.966 Da to tyrosine, serine, and threonine residues. The identified fully tryptic phosphopeptides were further examined by manually inspecting the MS/MS spectra to confirm identification of the correct peptide sequence and the correct sites of phosphorylation.

In vitro cell growth assay and in vivo tumor growth in the xenograft models

At 48 hr post-lentivirus infection, cells were seeded at the density of 4×10^3 cells/well in the 96-well plate with phenol red-free RPMI 1640 medium containing 5% charcoal-stripped serum and allowed to attach to the plate overnight. The cells were then treated with or without 10 ng/ml EGF for the indicated time. Growth was monitored using WST-1 (Roche) according to the manufacturer's instructions. The data were expressed as the average of normalized triplet samples (mean ± SD). The Student's t test was used to compare the statistic differences between groups. The tumor growth in the SCID/nude mice were carried out as described previously (Craft et al., 1999; Long et al., 2000). All procedures involving animals were approved by IACUC of the University of Maryland. To obtain the hormone-refractory tumors used in the study, tumor cells were allowed to grow in ten intact male mice for 4–10

weeks, respectively, to reach the sizes around 500 mm^3. The mice were then divided into two groups. One group was sacrificed and tumors were collected; another group were castrated and the tumor remission followed within a few weeks after castration. Subsequently, the tumors were allowed to regrow back to the size around 500 mm^3. Two or three independent hormone-refractory tumors were collected and subjected to the subsequent analysis along with their hormone-sensitive counterparts.

Immunohistochemical analysis

Two intermediate-density prostate tissue arrays were prepared by the NYU Cooperative Prostate Cancer Tissue Resource and consisted of a total 156 cases (four cores per case) including 18 hormone-resistant (HR) and 18 hormone-naive (HN) transurethral resection (TURP) specimens of prostate from patients with clinically advanced prostate cancer, 73 cases of HN prostate cancer tissue (Gleason sum 6–10) from the radical prostatectomy specimens of patients with clinically localized prostate cancer, and 47 cases of non-tumor-containing tissue from patients with benign prostatic hypertrophy. The determination of HN and HR was as follows: (1) patients who had earlier undergone surgical orchiectomy or medical hormone-suppressive therapy at least 6 months prior to the procedure were considered as HR; (2) patients who did not receive hormonal therapy prior to the TURP were considered as HN. Tissue specimens were from the archival paraffin block inventory of the NYU Cooperative Prostate Cancer Tissue Resource. All cases upon collection into the resource (under an IRB-approved protocol) had repeat pathology characterization of tissues and review of medical records.

The Vectastain Elite ABC Kit (Vector Laboratories) was used for immunohistochemical staining according to the protocol recommended by the manufacturer. Immunostaining was evaluated manually and graded using a two-score system based on intensity score (IS) and proportion score (PS) as described (Harvey et al., 1999). The immunoreactive score for each case was quantified by the average of four cores. The statistical analyses were carried out by using the SAS version 9.0 statistics software.

Supplemental data

The Supplemental Data for this article can be found online at http://www.cancercell.org/cgi/content/full/10/4/309/DC1/.

Acknowledgments

We would like to thank Drs. H.-J. Kung, M.-F. Lin, C.L. Sawyers, J.D. Fondell, C.W. Gregory, E. Wilson, and the NCI Cooperative Prostate Cancer Tissue Resource at NYU for providing the essential materials used in our study; Dr. P. Lee for validating our pathologic analysis; and Drs. D.K. Ann and Y. Yen for stimulating discussion. This work was supported in part by the NIH grants (CA85380, CA106504), DOD grants (DAMD17-03-1-0117, W81XWH-06-1-0199), and the Award from Prostate Cancer Foundation to Y.Q, the NIH Contract (No. NO1-CO-12400) to T.D.V, the NIH grant (NCI UO1- CA86772) to J.M., DOD Postdoctoral Fellowship (W81XWH-04-1-0015) to Z.G., and Pre-doctoral Fellowship (W81XWH-06-1-0005) to K.X.

Received: March 10, 2006
Revised: June 20, 2006
Accepted: August 28, 2006
Published: October 16, 2006

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