HER2/neu kinase-dependent modulation of androgen receptor function through effects on DNA binding and stability

Ingo K. Mellinghoff,[1,2] Igor Vivanco,[4] Andrew Kwon,[2] Chris Tran,[5] John Wongvipat,[5] and Charles L. Sawyers[1,2,3,4,5,*]

[1]Department of Medicine
[2]Department of Molecular and Medical Pharmacology
[3]Department of Urology
[4]Molecular Biology Institute
[5]Howard Hughes Medical Institute
David Geffen School of Medicine at UCLA, Los Angeles, California 90095
*Correspondence: csawyers@mednet.ucla.edu

Summary

Given the role of the EGFR/HER2 family of tyrosine kinases in breast cancer, we dissected the molecular basis of EGFR/HER2 kinase signaling in prostate cancer. Using the small molecule dual EGFR/HER2 inhibitor PKI-166, we show that the biologic effects of EGFR/HER-2 pathway inhibition are caused by reduced AR transcriptional activity. Additional genetic and pharmacologic experiments show that this modulation of AR function is mediated by the HER2/ERBB3 pathway, not by EGFR. This HER2/ERBB3 signal stabilizes AR protein levels and optimizes binding of AR to promoter/enhancer regions of androgen-regulated genes. Surprisingly, the downstream signaling pathway responsible for these effects appears to involve kinases other than Akt. These data suggest that the HER2/ERBB3 pathway is a critical target in hormone-refractory prostate cancer.

Introduction

The androgen receptor (AR) is a ligand-dependent transcription factor which plays a central role in male sexual development and differentiation. In addition to this physiologic function, AR plays a prominent role in the pathogenesis of human prostate cancer. Carcinoma of the prostate is the most common malignancy affecting males in the U.S. Up to 30% of patients develop metastatic disease and receive treatment with GnRH analogs and AR antagonists to block the action of testicular and adrenal androgens. This "androgen ablation therapy" effectively inhibits tumor cell growth for a variable period of time but is universally followed by tumor regrowth despite castrate levels of androgens. This latter "hormone-refractory" state of the disease is characterized by expression of the androgen receptor (AR) and AR-regulated genes such as prostate specific antigen (PSA), suggesting that the AR pathway is reactivated (Isaacs and Isaacs, 2004).

Several lines of evidence have implicated the epidermal growth factor receptor (EGFR, also known as ERBB1) and its dimerization partner HER2 (also known as ERBB2) in prostate cancer progression. Epidermal growth factor (EGF), the natural ligand for EGFR, is capable of inducing AR transcriptional activity (Culig et al., 1994). Transforming growth factor α (TGF-α), another natural EGFR ligand, is frequently coexpressed with its receptor in hormone-refractory but not hormone-naïve prostate cancer, indicative of an autocrine growth-stimulatory loop (Scher et al., 1995). Forced overexpression of the HER2 kinase in prostate cancer cells enhances AR function and hormone-independent growth (Craft et al., 1999; Yeh et al., 1999). The relevance of this latter finding has been questioned by the subsequent examination of clinical samples. While some of these studies report a trend toward higher HER2 levels in hormone-refractory prostate cancer (Osman et al., 2001; Shi et al., 2001; Signoretti et al., 2000), others have found no increase in HER2 gene copy number or HER2 protein in hormone-refractory disease (Calvo et al., 2003; Savinainen et al., 2002). This contrasts to human breast cancer, where HER2 kinase activation has been shown to be biologically relevant and results from gene amplification and HER2 protein overexpression (Slamon et al., 1987). The disappointing activity of the EGFR inhibitor Gefitinib in recent prostate cancer trials (Blackledge, 2003) has further

SIGNIFICANCE

The androgen receptor (AR) is a critical determinant in the progression of human prostate cancer to a hormone-refractory state. AR function is determined by the availability of cognate ligand, but can be modulated by kinase pathways. We investigated the contribution of ErbB receptor tyrosine kinases to AR function using pharmacological and genetic approaches. We show that HER2, but not EGFR, signals are required for optimal AR function at limiting concentrations of androgen. Regulation of AR function by HER-2 occurs through effects on AR DNA binding and protein stability. These results shed new insights into androgen receptor regulation and suggest that future clinical trials of ErbB kinase inhibitor therapy in hormone-refractory prostate cancer must consider effects on the HER2/ERBB3/PI3-kinase pathway.

challenged the role of the EGFR/HER2 axis in hormone-refractory prostate cancer, and reinforces the need to understand in greater molecular detail how ERBB kinase signals modulate androgen receptor function.

Kinase pathways have been implicated as modulators of nuclear hormone receptor function (Shao and Lazar, 1999). In some instances, the substrates of these phosphorylation signals have been identified. Regulation of estrogen receptor (ER) function by MAPK, for example, occurs through phosphorylation of the receptor itself (Kato et al., 1995), as well as through phosphorylation of the p160 nuclear receptor coactivators AIB1 (Font de Mora and Brown, 2000) and TIF-2 (Lopez et al., 2001). The mechanisms through which kinase signals modulate AR function are currently unknown. In analogy to the estrogen receptor, TIF-2 phosphorylation by MAPK has been shown to mediate effects of EGF on AR function in transfection experiments (Gregory et al., 2004). Direct phosphorylation by MAPK of purified AR peptides has been observed in vitro (Yeh et al., 1999), but could not be confirmed in a subsequent mass spectrometric analysis of AR phosphorylation (Gioeli et al., 2002). Perhaps more importantly, it remains uncertain whether the EGFR/HER2-MAPK axis is a biologically relevant modulator of AR function.

In this study, we dissect the crosstalk between ERBB RTKs and AR function using a dual EGFR/HER2 kinase inhibitor with previously documented hormone-dependent bioactivity (Mellinghoff et al., 2002). Our results show that ERBB kinase signals are required for optimal AR function at physiologically low androgen concentrations. RNA interference experiments show that these signals are not mediated through EGFR, but rather through the association of HER2 with other members of the ERBB receptor family. Optimization of AR function through HER2 occurs through increased AR binding to its cognate DNA target regions and through protection of AR from ubiquitin-mediated degradation. Surprisingly, the downstream kinase responsible for these AR effects appears to be distinct from Akt. Our findings provide novel insights into AR function and suggest that successful application of ERBB kinase family inhibitors in hormone-refractory prostate cancer may require specific targeting of the HER2/ERBB3 heterodimer complex.

Results

ERBB kinase activity is required for full AR function

We previously reported that androgen modulates the ability of the ERBB-selective kinase inhibitor PKI-166 (Traxler et al., 2001) to inhibit prostate cancer growth in vivo. While tumors growing in castrate male animals were particularly sensitive to ERBB RTK inhibition, this sensitivity was lost following androgen supplementation (Mellinghoff et al., 2002). This observation raised the question whether ERBB RTKs support prostate cancer growth through regulation of the AR pathway. To address this question, we defined the contribution of ERBB signals to AR function over a wide range of androgen concentrations. These concentrations were chosen to represent not only androgen levels found in adult healthy men, but also reduced concentrations measured in human prostate cancer tissue in the setting of androgen ablation therapy (Mohler et al., 2004).

In our first set of experiments, AR function was measured using a transiently transfected luciferase reporter gene driven by an artificial promoter containing four androgen response elements (AREs). In cell lines expressing either endogenous (LAPC4, LNCaP) or transfected (COS-7) AR, pretreatment with PKI-166 (5 μM) impaired AR activation by the synthetic androgen R1881, and the degree of inhibition was invariably greatest at R1881 concentrations between 0.1 and 1 nM (Figure 1A).

Since transcriptional activity of nuclear hormone receptors is dependent on a specific promoter context, we extended the determination of AR activity from artificial promoters to the endogenous androgen regulated gene prostate-specific androgen (PSA). Similar to our reporter gene assays, the effect of PKI-166 on PSA expression in LAPC4 cells was maximal between 0.1 and 1 nM R1881 (Figure 1B, upper panel). Inhibition of PSA expression by PKI-166 was also observed in human prostate cancer xenografts growing subcutaneously in *SCID* mice. Since mice do not express PSA, the ratio between serum PSA and tumor volume ("PSA index") approximates AR activity in the implanted tumors (Gleave et al., 1992). Treatment of mice for two weeks with PKI-166 reduced the PSA index in female and castrated male mice, but not in castrated male mice receiving dihydrotestosterone replacement (Figure 1B, lower panel).

To examine the interplay between androgen receptor and ERBB RTKs in nonmalignant tissue, we examined the effects of PKI-166 on the development of male murine urogenital tissue. Daily treatment with PKI-166 was initiated at the beginning of the growth spurt for prostate and seminal vesicles (postnatal day 20) (McKinney and Desjardins, 1973) and continued for two weeks. Compared to vehicle treated littermate controls, the wet weight of prostate/seminal vesicles from PKI-166 treated mice was reduced by approximately 35%. Exogenous androgen supplementation during the period of PKI-166 treatment rescued the effect of PKI-166 on the growth of prostate/seminal vesicle (Figure 1C). Prostatic epithelial differentiation was not affected by PKI-166 and overall gland morphology appeared normal (data not shown). This antiandrogen-like effect of PKI-166 was not due to endocrine effects, as serum testosterone levels were unaffected by PKI-166 (Figure 1C, insert). This phenotype was reminiscent of the impaired growth of fetal urogenital sinuses from IGF-I and from IGF-R1 knockout mice grafted beneath the renal capsule of athymic mice (Hayward and Cunha, 2000). In summary, these results indicate that ERBB-mediated signals contribute to AR function in malignant and nonmalignant tissue, and that this contribution is greatest when the androgen concentrations are not saturating.

The antiandrogen properties of PKI-166 are explained by HER2, not EGFR inhibition

The ERBB receptor kinases includes four family members: EGFR, HER2, ERBB3, and ERBB4. Despite the lack of a natural ligand, HER2 plays a prominent role within the ERBB signaling network as a preferred heterodimerization partner for other ligand-bound ERBBs (Olayioye et al., 2000). As a first approximation to define this signaling network in prostate cancer, we analyzed ERBB gene expression profiles of seven human prostate cancer xenografts (Figure 2A, left panel). In this group of samples, normalized RNA expression of HER2 (mean $\pm$ SEM 2.74 $\pm$ 0.23) and ERBB3 (1.83 $\pm$ 0.32) was significantly higher than expression of EGFR (0.78 $\pm$ 0.17) and ERBB4 (0.09 $\pm$ 0.08). Relative levels of ERBB gene expression in these xenograft tumors correlated closely with relative levels of ERBB protein expression in the prostate cancer cell lines derived from these xenografts (Figure 2A, right panel).

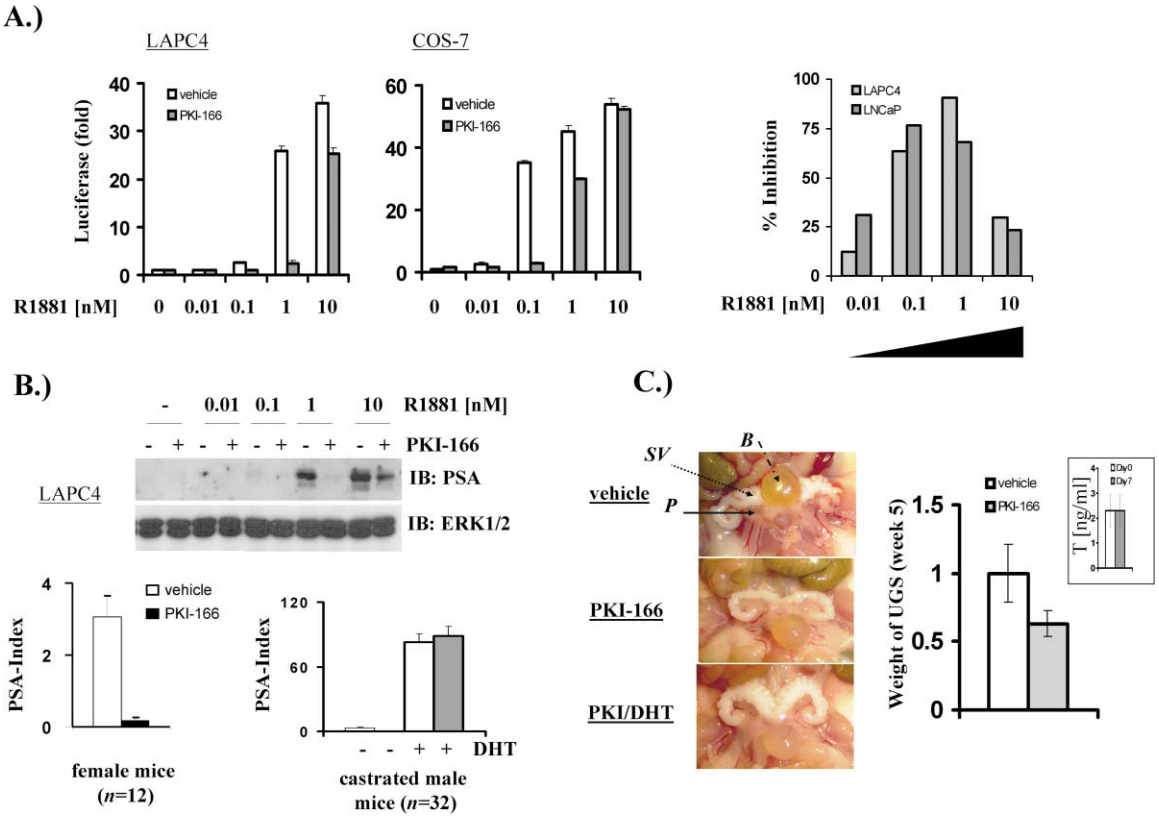

Figure 1. PKI-166 inhibits androgen receptor function

A: Effect of PKI-166 on AR reporter gene activity. Increasing amounts of the synthetic androgen R1881 were added to cells transfected with the AR reporter gene 4xARE-luciferase in the presence or absence of the dual EGFR/HER2 kinase inhibitor PKI-166. LAPC4 cells express endogenous AR; COS-7 cells were cotransfected with pCMV-AR. The right panel shows % mean inhibition of AR-reporter activity by PKI-166 in the prostate cancer cell lines LAPC4 and LNCaP.
B: Effect of PKI-166 on protein expression of the endogenous androgen-regulated gene prostate specific antigen (PSA). Upper panel: increasing amounts of R1881 were added to LAPC4 cells growing in hormone-depleted media, and PSA induction was determined after three days by immunoblotting of whole cell lysates. Lower panel: *SCID* mice bearing established subcutaneous human prostate cancer xenografts were treated for two weeks with PKI-166 (100 mg/kg) or vehicle. The PSA index was determined as ratio between tumor volume and serum PSA measured by ELISA. PKI-166 was administered daily by gavage, and Dihydrotestosterone (DHT) was administered as slow-release pellets.
C: Effect of PKI-166 on development of the male urogenital system in FVB inbred mice. Intact male mice were treated with PKI-166 (100 mg/kg) or vehicle between postnatal day 20 and 34. Dihydrotestosterone (DHT) was administered as slow-release pellets. At the end of the experiment, the male urogenital tract (P: prostate, SV: seminal vesicle, B: bladder) was photographed in situ. Shown are the wet weights (mean ± SD) from PKI-166 treated mice compared to vehicle-treated mice (n = 6 per group). Inset, serum testosterone levels (mean ± SD) in six adult male mice before and during treatment with PKI-166.

Although PKI-166 is known primarily as an EGFR inhibitor (Mendelsohn, 2001), it has activity against HER2 at 10-fold higher concentrations (Holbro et al., 2003; Traxler et al., 2001). We previously demonstrated (Mellinghoff et al., 2002) that both kinases are inhibited by the doses used in all of the experiments performed in Figure 1. To dissect which members of the ERBB signaling network contribute to AR function, we employed three approaches: (1) drugs with a wider differential in the inhibitory concentrations (IC_{50}s) required to block EGFR and HER2 RTK activity, (2) an EGFR-negative cell line, and (3) genetic silencing of individual ERBB family members using RNA interference. The quinazoline PD153035 (Fry et al., 1994) is an EGFR kinase inhibitor that also inhibits the HER2 kinase, but only at 100-fold higher drug concentrations than the EGFR RTK. At drug concentrations that completely inhibit the EGFR RTK, PD153035 had no effect on R1881-induced AR function. A marked reduction of AR function, however, was noted at drug concentrations which also inhibit HER2 (Figure 2B). This data indicates that EGFR RTK inhibition is not sufficient to impair AR function.

To determine whether EGFR is required to mediate the effect of ERBB kinase inhibitors on AR function, we performed AR reporter assays in a 3T3 subline which does not express functional EGFR (Pruss and Herschman, 1977). Inhibition of AR function by PKI-166 in these cells was very similar in magnitude (Figure 2C) to inhibition of AR function in EGFR-expressing cells (Figure 1A). These experiments suggested that HER2, but not EGFR, contributes to AR function at low androgen levels. To obtain genetic evidence for this model, we used RNA interference to transiently and selectively knock down expression levels of either EGFR and/or HER2. This analysis was performed in three different cell lines (Figure 2D) using two different target sequences for each gene (Supplemental Figure S1A) to minimize gene nonspecific effects of interfering RNAs (Moss and Taylor, 2003). EGFR knockdown had no significant effect on AR activity, whereas genetic silencing of HER2 by RNA interference mirrored the effect of PKI-166 (Figure 2D). Control experiments established that the RNAi silencing was specific for each ERBB kinase isoform (Supplemental Figure S1A). In summary, these experi-

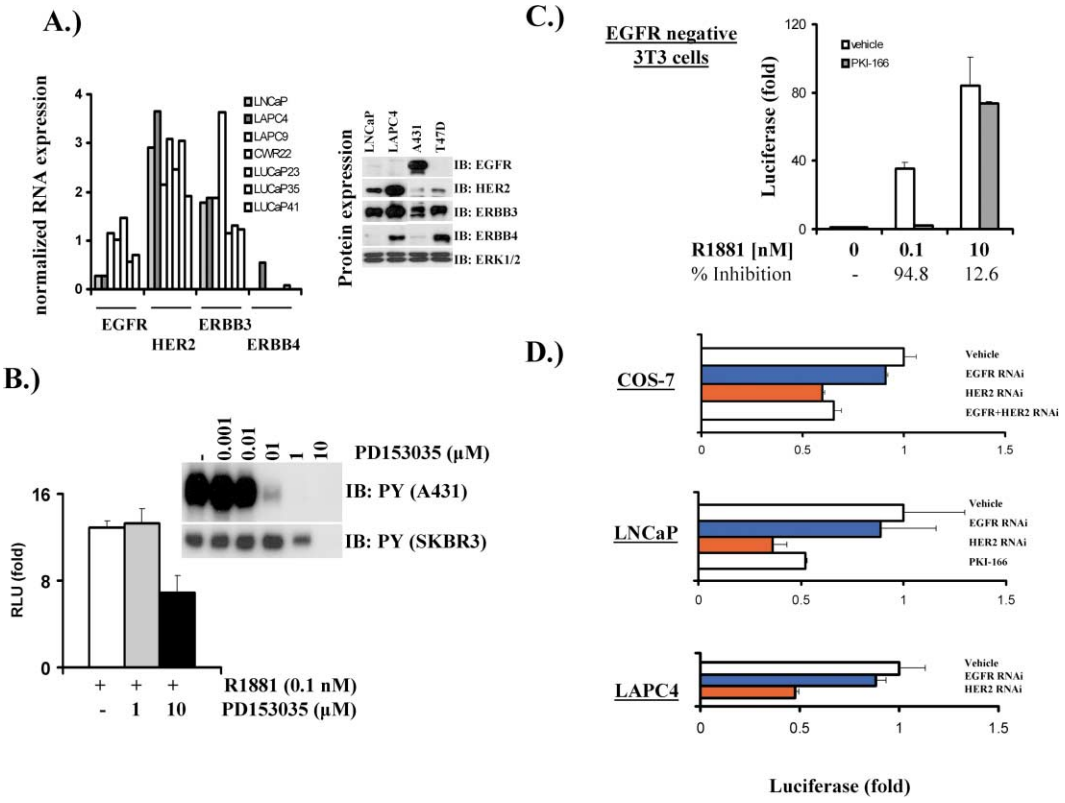

Figure 2. The HER2 kinase, not EGFR, is critical for AR function

A: Expression of ERBB receptors in human prostate cancer. Left panel: normalized RNA expression values for ERBB receptor family members (EGFR, HER2, ERBB3, ERBB4) in seven human prostate cancer xenograft tumors (LNCaP, LAPC4, LAPC9, CWR22, LUCaP23, LUCaP35, LUCap41). Right panel: ERBB protein expression levels in the prostate cancer cell lines LNCaP and LAPC4. Whole cell lysates from A431 and T47D cells were loaded as positive controls for the EGFR and ERBB4 antibodies, respectively.

B: Effect of the EGFR-selective small molecule kinase inhibitor PD153035 on R1881-induced AR reporter gene activity (4xARE-Lux) in COS-7 cells. Inset: IC_{50}s for inhibition of EGFR and HER2 autophosphorylation by PD153035 as determined by phosphotyrosine immunoblotting in A431 (EGFR) and SKBR3 (HER2) cells.

C: Effect of PKI-166 on AR reporter gene activity (4xARE-Lux) in EGFR negative 3T3 cells.

D: Effect of isoform-specific siRNAs against EGFR and HER2 on ligand-induced AR reporter gene activity in the three cell lines, COS-7, LNCaP, and LAPC4. Results are representative for RNAis targeting two different sequences of EGFR and HER2.

ments indicated that the HER2 kinase is the relevant target of PKI-166 in terms of AR regulation. In contrast, EGFR plays a minor, if any, role.

To further validate the significance of the HER2 kinase for AR function and prostate cancer growth, we examined the effects of stable HER2 RNAi knockdown on growth and AR function in human prostate cancer cell lines. As predicted by our results with PKI-166, knockdown of HER2 resulted in impaired growth (Figure 3A) and AR activity (Figure 3B). Importantly, the growth inhibitory effects of HER2 knockdown could be rescued by raising the concentration of R1881 from 0.5 to 5 nM (Figure 3C). Since HER2 is an orphan receptor and recruits other ERBB family members as part of its activation platform, we reasoned that PKI-166 must inhibit signals originating from HER2/ERBB3 and/or HER2/ERBB4 heterodimers. Indeed, treatment of LNCaP and LAPC4 cells with the ERBB3/ERBB4 ligand heregulin-β1 resulted in PKI-166 reversible tyrosine phosphorylation of proteins of about 180 kDa (Figure 3D), representing phosphorylated ERBB2 and ERBB3 (Sliwkowski et al., 1994; Holbro et al., 2003). In summary, these results suggest that the effects of PKI-166 on AR function are EGFR-independent and may be mediated through the HER2/ERBB3 axis. These data do not exclude a more general role for EGFR in prostate cancer growth, particularly since EGFR expression levels in these two cell line models (LNCaP and LAPC4) are lower than in other prostate cancer models (Figure 2A).

Regulation of AR function through Akt-dependent and -independent signals

Since HER2/ERBB3 and HER2/ERBB4 heterodimers signal primarily through the PI3Kinase (PI3K) pathway (Carraway et al., 1995), we examined in greater detail the role of this pathway for AR function. Previous studies have yielded controversial results as to whether the serine/threonine kinase Akt, a main effector of the PI3K pathway, enhances (Wen et al., 2000; Lin et al., 2003; Nan et al., 2003) or impairs (Lin et al., 2001, 2002, 2003) AR function and stability. Akt has been suggested to directly phosphorylate AR at the Serine 213 and Serine 791 (Wen et al., 2000; Lin et al., 2001), but no evidence for phosphorylation at these sites was seen by mass spectrometric examination (Gioeli et al., 2002; Wong et al., 2004).

Inhibition of the lipid kinase PI3K with LY294002 (12.5 μM)

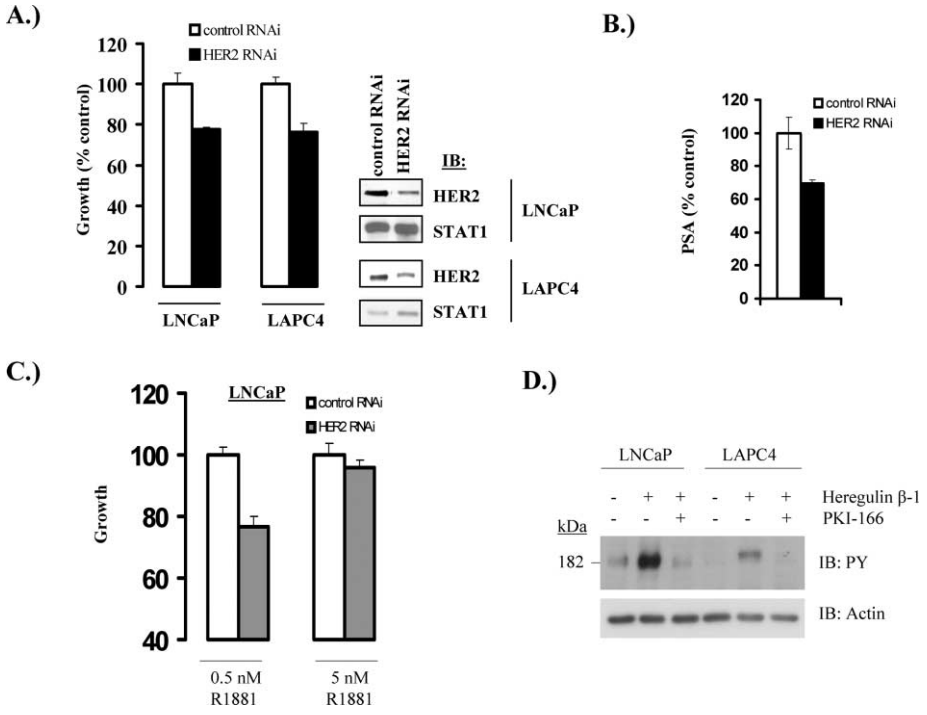

Figure 3. HER2 kinase regulates prostate cancer growth through its effects on AR function

A: Effect of HER2-specific hairpin RNAi on growth of LNCaP and LAPC4 cells. Cells were grown in charcoal stripped (CS) media supplemented with 0.5 nM R1881 and counted in quadruplets. Right panel, HER2 immunoblot of whole cell lysates from each cell line. STAT-1 was used as loading control.

B: Effect of HER2-specific hairpin RNAi on PSA production of LNCaP cells. PSA concentration was determined in the supernatatant of LNCaP cells growing in CS media supplemented with 0.5 nM R1881. PSA values were normalized per cell number.

C: Growth of LNCaP-HER2 RNAi cells and LNCaP-control RNAi cells in CS media supplemented with either 0.5 or 5 nM R1881.

D: Phosphotyrosine immunoblot of LNCaP and LAPC4 cells stimulated for 30 min with Heregulin β-1 (50 ng/ml) in the presence or absence of PKI-166.

impaired the induction of AR function by 0.1 nM R1881 to a similar degree as PKI-166 (Figure 4A, left panel). Genetic inhibition of PI3K signaling by overexpression of the phosphatase PTEN similarly impaired AR function and lowered AR protein levels (Figure 4A, right panel). Overexpression of a constitutively active, myristoylated Akt (myr-Akt) allele in LAPC4 prostate cancer cells markedly raised AR protein levels and PSA production in vitro and in vivo (Figure 4B). In summary, these experiments validate the PI3K/Akt signaling axis as an important positive regulator of AR function and stability.

We next asked whether the effects of PKI-166 on AR function, demonstrated above to be HER2/ERBB3-dependent, are mediated through Akt. Surprisingly, overexpression of myr-Akt in LAPC4 cells could not rescue the inhibitory effects of PKI-166 on AR protein levels and PSA production (Figure 4C, upper panel). Similar results were observed in an isogenic 3T3 cell line pair stably expressing vector control versus myr-Akt and transiently transfected with AR and an androgen-dependent reporter construct (data not shown). Immunoblots using a phospho-Akt antibody showed that PKI-166 has no effect on phospho-Akt levels in myr-Akt-expressing cells (Figure 4C, lower panel), ruling out the possibility that this failure to rescue is due to direct inhibition of Akt. Since myr-Akt may not replace all the functions of endogenously activated Akt, it remains possible that Akt plays a role in the HER2/ERBB3 signal to AR.

We next addressed whether AR itself is a substrate in the kinase pathway modulated by PKI-166. Based on sequence analysis for kinase consensus motifs (Blom et al., 1999), the androgen receptor contains over 40 predicted phosphorylation sites. Seven of these sites have been shown to be phosphorylated in a recent mass spectrometric analysis of AR (Gioeli et al., 2002). Using a commercially available phosphospecific antibody to Serine 81, one of the sites most intensely phosphorylated in response to ligand, we observed a consistent reduction in AR phosphorylation at this residue when cells were treated with PKI-166 (Figure 4D). Interestingly, this effect of PKI-166 was less pronounced at higher R1881 concentrations, correlating with the dose-dependent effects of androgen on the ability of PKI-166 to modulate AR function (Figure 1A). This observation establishes that AR is a substrate of this HER2 regulated kinase pathway. The identity of the direct AR kinase is unknown, but appears unlikely to be Akt, since amino acids surrounding Serine 81 do not resemble an Akt consensus site.

HER2 kinase stabilizes androgen receptor protein

Kinase pathways have been shown to modulate all aspects of nuclear hormone receptor function (Shao and Lazar, 1999). To identify the mechanism of AR regulation by HER2, we examined the effects of PKI-166 on AR ligand binding using saturation binding assays. This is a critical first experiment, because interference with ligand binding would be the simplest explanation for the effects of PKI-166 on R1881-induced AR phosphorylation shown above in Figure 4D. However, in both LAPC4 and LNCaP prostate cancer cells, PKI-166 pretreatment only modestly reduced the high binding affinity of AR (Supplemental Figure S2A). We next asked whether HER2 kinase inhibition impaired ligand-induced translocation of AR from the cytosolic to the nuclear compartment. In the absence of ligand, AR was predominantly found in the cytoplasm using both immunofluorescence and cell fractionation assays. Treatment with 1 nM R1881 resulted in translocation of AR to the nuclear compartment within 60 min and was not affected by treatment with PKI-166 (Supplemental Figure S2B).

During our cell fractionation experiments, we observed decreased AR protein levels in both nuclear and cytosolic compartments at later time points (72 hr). We therefore examined the

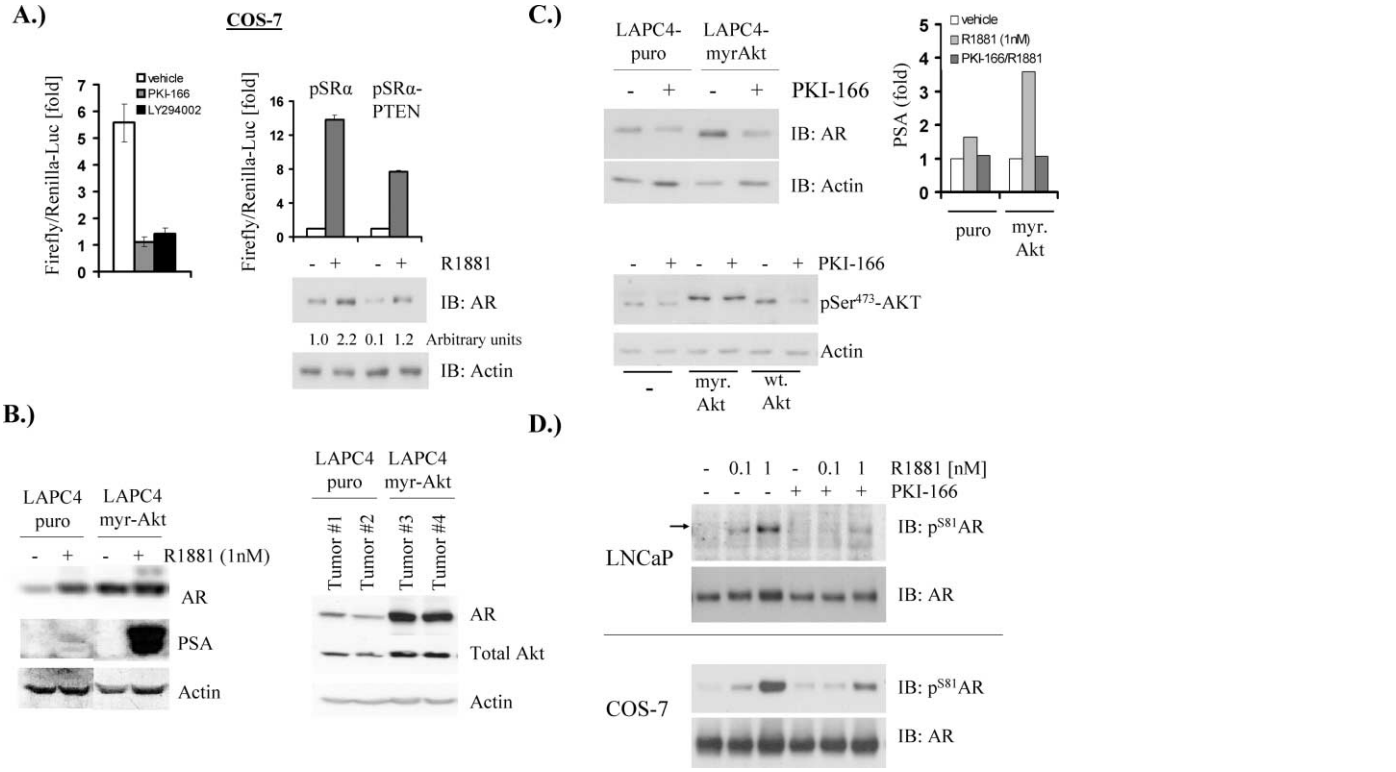

Figure 4. Role of the PI3Kinase/Akt pathway in AR function

A: Left panel: effect of the PI3K inhibitor LY294002 on AR function. COS-7 cells transiently transfected with 4xARE-Luc, pCMV-AR, and renilla-luciferase were treated for 30 min with LY294002 or PKI-166 prior to induction with 0.1 nM R1881. Right panel: effect of the phosphatase PTEN on AR function. COS-7 cells were transfected with 4xARE-Luc, pCMV-AR, renilla-luciferase, and pSRα or pSRα-PTEN and reporter activity induced with 0.1 nM R1881. Immunoblots show AR levels in lysates from one representative sample for each reporter assay triplicate.

B: Left panel: expression of AR and PSA in parental LAPC4 cells and an isogenic subline expressing myristoylated Akt (myr-Akt). Cells were grown in CS media supplemented with vehicle or 1 nM R1881 for 36 hr prior to lysis and immunoblotting. Right panel: expression of AR and Akt in parental LAPC4 cells and myr-Akt LAPC4 cells growing as xenograft tumors in intact male *SCID* mice.

C: Upper panel: LAPC4 cells growing in CS media supplemented with 0.5 nM R1881 were treated with PKI-166 for 72 hr. AR levels were determined by immunoblotting of whole cell lysates. PSA values reflect secreted PSA (measured by ELISA) per cell. Lower panel: effect of PKI-166 on wild-type endogenous Akt, myr-Akt, and overexpressed wild-type Akt in stable 3T3 sublines. Cells were lysed 6 hr after addition of PKI-166 or vehicle.

D: PKI-166 impairs R1881-induced phosphorylation of AR at Ser81. LNCaP cells and COS-7 cells transfected with pCMV-AR were treated with PKI-166 for 30 min prior to induction with R1881. Cells were lysed 6 hr after R1881-induction and probed with a phosphospecific antibody for AR-Ser81.

effects of HER2 kinase inhibition on AR protein levels in greater detail in whole cell lysates. Treatment of LAPC4 cells for three days with the synthetic androgen R1881 led to a dose-dependent increase in AR protein levels (Figure 5A, upper panel), consistent with ligand-induced receptor stabilization (Kemppainen et al., 1992). Simultaneous treatment with PKI-166 markedly reduced AR levels at nonsaturating androgen concentrations. The effect of PKI-166 on AR protein levels was also observed in three breast cancer cell lines expressing endogenous wild-type AR (Figure 5A, lower panel), suggesting a more general phenomenon of AR regulation.

We next asked whether the effect of PKI-166 on AR protein levels resulted from decreased AR transcription or destabilization of the receptor protein. Using quantitative RT-PCR, we found no effect of PKI-166 on AR transcript levels, whereas PSA transcript levels were markedly reduced (Figure 5B). These results indicate that regulation of AR levels by HER2 occurs at a posttranscriptional level. Pulse-chase experiments confirmed that inhibition of HER2 by PKI-166 accelerated the degradation of the androgen receptor (Figure 5C, top panel). Similar to other steroid hormone receptors (Nawaz et al., 1999a; Lange et al., 2000), AR is a substrate of the ubiquitin-proteasome pathway (Sheflin et al., 2000). Cotransfection of AR with HA-epitope-tagged ubiquitin into COS-7 cells confirmed that AR is, indeed, ubiquitinated, and that the degree of ubiquitination is greatest at low androgen concentrations and enhanced by treatment with PKI-166 (Figure 5C, middle panel). In summary, these data indicate that AR protein levels are regulated by a HER2/ERBB3 kinase signal. Consistent with this model, cotransfection of HER2, but not EGFR, raised the levels of AR transiently transfected into COS-7 cells (Figure 5D).

HER2 enhances binding of AR to AREs in androgen-responsive genes

If modulation of AR protein levels is the primary mechanism by which the HER2/ERBB3 axis regulates AR function, we hypothesized that forced elevation of AR levels should rescue the effect of PKI-166 on AR function. We tested this hypothesis using two

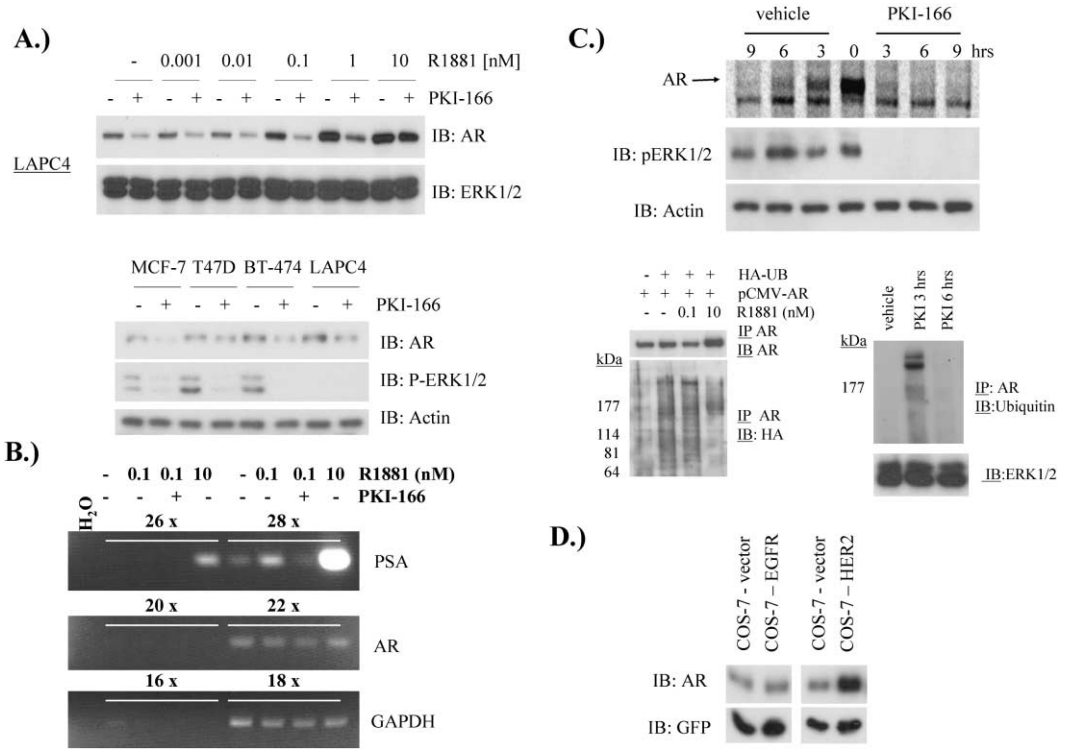

Figure 5. HER2 kinase stabilizes AR protein levels

A: Effect of PKI-166 on AR protein levels. Upper panel: immunoblot analysis of LAPC4 prostate cancer cells treated for two days with increasing concentrations of R1881 in the presence or absence of PKI-166. Lower panel: immunoblot analysis of three breast cancer cell lines (MCF7, T47D, BT474) growing for two days in CS media in the presence or absence of PKI-166.

B: Semiquantitative RT-PCR analysis for AR, PSA, and GAPDH mRNA in LAPC4 cells treated for two days with R1881 in the presence or absence of PKI-166.

C: Upper panel: pulse-chase analysis of androgen receptor stability in COS-7 cells transfected with pCMV-AR. Lower panel, left: COS-7 cells were transfected with pCMV-AR and HA-tagged ubiquitin and treated with R1881 for 36 hr. Anti-AR immunoprecipitates from whole cell lysates were probed with anti-HA and anti-AR antibodies. Lower panel, right: COS-7 cells were transfected with pCMV-AR and treated with PKI-166 for three or six hours. Whole cell lysates were subjected to anti-AR immunoprecipitation followed by immunoblotting with a polyclonal anti-ubiquitin antibody.

D: COS-7 cells were transfected with pCMV-AR and expression vectors for EGFR or HER2 for 36 hr and AR levels determined by immunoblotting in whole cell lysates.

different approaches: (1) inhibition of proteasomal AR degradation using the proteasome inhibitor lactacystin (Fenteany and Schreiber, 1998), and (2) forced overexpression of AR by lentiviral expression. Pretreatment of COS-7 cells with lactacystin rescued the effects of PKI-166 on AR protein levels (Figure 6A, upper panel), supporting the notion that HER2 stabilizes AR protein levels by protecting it from ubiquitin-mediated degradation. This increase in AR protein levels, however, was not sufficient to restore AR function in the presence of PKI-166 (Figure 6A, lane 8 versus lane 4). Similar to our lactacystin experiment, forced overexpression of AR by lentiviral infection was not sufficient to rescue the inhibitory effects of PKI-166 on AR function (Figure 6B, lower panel). In summary, these results suggest that the HER2/ERBB3 axis regulates AR function through additional mechanisms beyond protein stabilization.

Since regulation of DNA binding by phosphorylation has been documented for various transcription factors (Yamamoto et al., 1988; Prywes et al., 1988), we examined ligand-induced recruitment of AR to its cognate DNA target sequences ("AREs" or androgen-response elements) using chromatin immunoprecipitation. The 5′-regulatory regions of the PSA and KLK-2 genes were chosen as DNA target sequences for these experiments, because they contain several AREs in the promoter and enhancer regions (Cleutjens et al., 1996, 1997; Mitchell et al., 2000). As previously reported, treatment of LNCaP cells with R1881 resulted in rapid recruitment of AR to the PSA enhancer region (Louie et al., 2003; Shang et al., 2002) (Figure 6B). Recruitment to the PSA-promoter was only marginally induced by 0.1 nM R1881. AR recruitment to the PSA enhancer was almost completely blocked by pretreatment with PKI-166. Identical results were obtained when AR recruitment to the KLK-2 enhancer was examined. Recruitment of RNA polymerase II to the gene regulatory region of the PSA and KLK-2 genes corresponded closely with AR binding, suggesting that recruitment of AR is the rate-limiting step in assembly of a functional transcription complex. Of note, chromatin immunoprecipitation experiments were performed within 1 hr of PKI-166 exposure, prior to any measurable effects on AR protein levels.

Since our biological and transcriptional experiments showed that saturating levels of androgen rescue the effects of HER2 inhibition on AR function, we predicted that androgen should also rescue the effect on DNA binding. Indeed, when R1881 concentrations were raised from 0.1 nM to 10 nM, PKI-166 no

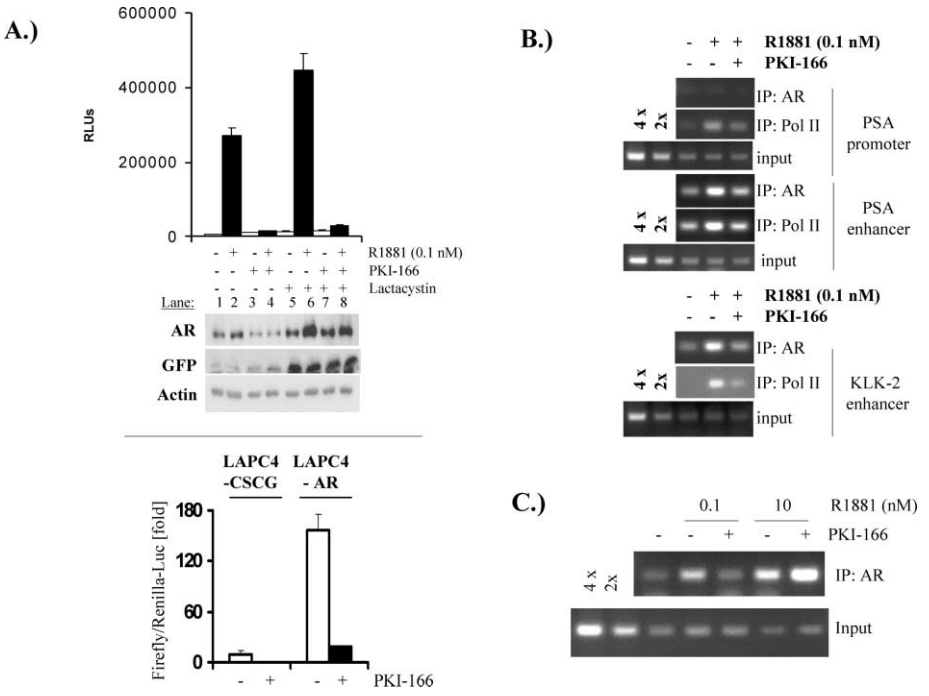

Figure 6. PKI-166 impairs binding of AR to cognate DNA target sequences

A: Upper panel: concurrent analysis of AR reporter gene activity (4xARE-Lux) and AR protein levels in lysates from transfected COS-7 cells treated with PKI-166 in the presence or absence of the proteasome inhibitor lactacystin. Pretreatment with lactacystin started six hours prior to addition of PKI-166. Lower panel: LAPC4 cells stably expressing CSCG-vector or CSCG-AR were transfected with 4xARE-Luc and renilla luciferase and treated with PKI-166 or vehicle prior to induction with 0.1 nM R1881.

B: Chromatin immunoprecipitation of AR and RNA polymerase II (Pol II) in LNCaP cells treated for one hour with 0.1 nM R1881 in the presence or absence of PKI-166. Linearity of the amplification reaction was monitored by using three different volumes (1×, 2×, 4×) of input DNA from vehicle-treated samples. Shown is DNA occupancy at the PSA promoter, PSA enhancer, and KLK-2 enhancer.

C: Chromatin immunoprecipitation analysis of AR recruitment to the PSA enhancer in LNCaP cells treated for 60 min with R1881 in the presence or absence of PKI-166.

longer impaired the immediate androgen-induced recruitment to the PSA enhancer (Figure 6C).

Discussion

In this study, we have defined a HER2-dependent signal that is critical for AR function at low androgen concentrations. Our finding that this signal is EGFR-independent shifts the emphasis from the EGFR/HER2-MAPK axis to the HER2/ERBB3 axis. Further support for a role of the HER2/ERBB3 axis in hormone-refractory prostate cancer stems from recent preclinical studies. Interruption of HER2/ERBB3 heterodimerization using the monoclonal antibody 2C4 potently inhibited the growth of hormone-refractory prostate cancer xenografts (Agus et al., 2002), whereas Trastuzumab, which does not block HER2/ERBB3 dimerization, had minimal activity (Agus et al., 1999). Our data showing that HER2 but not EGFR is responsible for this AR signal suggests that pathways unique to the HER2/ERBB3 heterodimer (PI3K, Akt, others) are more likely mediators than Raf/MEK/Erk. This conclusion is further supported by the analogous effects of PI3K inhibitors on AR function.

The precise details of how the HER2/ERBB3 kinase pathway modulates AR function remain to be elucidated. We currently favor a model in which AR is a substrate of a HER2-regulated kinase cascade. The strongest evidence in favor of this model is PKI-166-reversible phosphorylation of AR at Serine 81 in response to androgen. Modulation of phosphorylation at this site correlates precisely with the effects of PKI-166 on AR stability and transcriptional activity as well as rescue by higher concentrations of R1881. Much more work is needed to define the kinase directly responsible for AR phosphorylation at this residue, and whether AR Serine 81 is solely responsible for these effects. Serine81 occurs in the context of a Protein Kinase C consensus site; however, PKC inhibitors failed to impair AR phosphorylation at this residue (Gioeli et al., 2002). Furthermore, mutagenesis studies at this site (AR S81A), at first glance, failed to phenocopy the biologic effects of PKI-166 treatment. Collectively, these data suggest a complex mode of AR regulation by one or more kinases downstream of HER2/ERBB3. Our reconstitution studies with Akt, which fail to rescue the effects of PKI-166 on AR function, cast some doubt as to whether the PI3K/Akt pathway is the effector of this signal.

Besides the androgen receptor itself, other members of the AR signaling pathway may be substrates of HER2/ERBB3-regulated kinase signals. There is ample precedent for phosphorylation of steroid hormone receptor coactivators and corepressors (Font de Mora and Brown, 2000; Janknecht and Nordheim, 1996; Hong and Privalsky, 2000; Lopez et al., 2001; Rowan et al., 2000). To date, MEK-Erk kinases have been tightly implicated in modulating coregulator function, but there is no knowledge about such regulation by PI3K-related pathways. Further experiments are required to determine if HER2/ERBB3 signals can affect AR function through phosphorylation of coregulators.

We defined two properties of AR regulation by the HER2 kinase, DNA binding and protein stability (Figure 7). There are a number of examples in the hormone receptor field showing regulation of DNA binding by phosphorylation (Arnold and Notides, 1995; Bhat et al., 1994; Chen et al., 1999; Denner et al., 1989; Katz et al., 1995; Rangarajan et al., 1992; Tzagarakis-Foster and Privalsky, 1998; Sugawara et al., 1994; Hirata et al., 1993). Further experiments will determine whether the effect of HER2 on DNA binding is due to effects on AR dimerization, increased dissociation of heat-shock proteins with unmasking of the DNA binding sites, and/or altered assembly with coactivator proteins which stabilize the formation of the AR transcription complex. There is also increasing evidence for a role of the ubiquitin-proteasome degradation system in transcriptional regulation (Lipford and Deshaies, 2003). The ubiquitin-ligase E-6

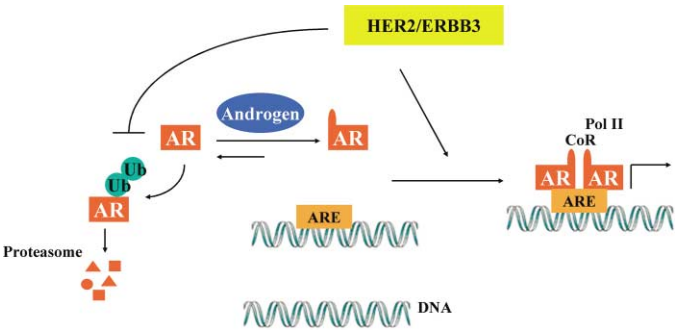

Figure 7. Model for the dual effects of the HER2/ERBB3/PI3K signal on androgen receptor protein stability and DNA binding

The HER2/ERB3 axis provides signals to AR that enhances its DNA binding to ARE target sequences and protect AR from proteasome-mediated degradation. CoR, coregulators; PolII, RNA-Polymerase II; Ub, ubiquitin.

associated protein, for example, has been shown to function as a coactivator for the steroid receptor superfamily (Nawaz et al., 1999b). Whether a more complex model accounts for the effects of the Her2/ERBB3-PI3K signal on AR function will await detailed mapping of kinase-dependent phosphorylation changes in all members of the AR pathway.

The androgen receptor has emerged as key molecular determinant in the progression of human prostate cancer to a hormone-refractory state. Increased AR expression is frequently observed in hormone-refractory tumors (Edwards et al., 2003) and is sufficient to convert prostate cancer growth from a hormone-sensitive to a hormone-refractory stage (Chen et al., 2004). Our results show that small molecule inhibitors of the ERBB2 RTK potently antagonize AR function and may add to currently available AR antagonists which are limited by their potency and partial agonism (Taplin et al., 1999; Chen et al., 2004). By precisely defining the kinases and phosphorylation sites on AR and/or AR-associated proteins regulated by this HER2 signal, we envision developing reagents that may identify the subset of patients most likely to benefit from anti-HER2 therapy.

Experimental procedures

Reagents

The LAPC4 cell line was established from the LAPC4 human prostate cancer xenograft (Klein et al., 1997). The human prostate cancer cell line LNCaP, the human breast cancer cell lines T-47D, MCF-7, BT-474, and SKBR3, and COS-7 monkey kidney cells were obtained from the American Type Culture collection. NR6 cells were kindly provided by Dr. Herschman (UCLA), HA-tagged ubiquitin by Dr. Bohmann (University of Rochester), and pCDNA S81A-AR by Dr. Gioeli (University of Virginia). PKI-166 was provided by Drs. Buchdunger and Traxler (Novartis Pharma AG) and used in vitro at a concentration of 5 μM. Commercially available reagents included: methyltrienolone (R1881) (PerkinElmer, Boston, Massachusetts); heregulin β-1 (Lab Vision, Fremont, California); LY294002, SB203580, SP60012, and lactacystin (Calbiochem, La Jolla, California); antibodies to EGFR, ERBB3, ERBB4, AR, phospho-ERK1/2, PSA, and ubiquitin (Santa Cruz Biotechnology, Santa Cruz, California); antibodies to AR, phospho-Ser81 AR, and phosphotyrosine (Upstate, Lake Placid, New York); and antibodies to HER2 (Oncogene Sciences, San Diego, California).

Reporter assays

Cells were transfected with the indicated expression plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, California). PKI-166 was added six hours after transfection and 30 min prior to addition of R1881. Luciferase activity was assayed in triplicates 36 hr after transfection. To control for general effects on transcription, constitutive expression plasmids for β-galactosidase or renilla luciferase were cotransfected in all reporter assays and Luciferase values represent ratios of firefly luciferase/β-galactosidase or firefly luciferase/renilla luciferase.

Animal experiments

Mice were maintained in a defined colony and treated following the guidelines of the UCLA Animal Research Committee. PKI-166 was administered daily by gavage at a dose of 100 mg/kg. Testosterone pellets (Innovative Research of America, Sarasota, Florida) were implanted subcutaneously.

Gene expression profiling and RT-PCR

Normalized RNA expression values for ERBB family members was surveyed in a previously described dataset generated from seven different human prostate cancer xenograft models (Chen et al., 2004). RT-PCR was performed as previously described (Chen et al., 2004).

AR translocation, ligand binding, and stability

Immunofluorescence, cell fractionation, saturation binding, and pulse-chase assays were performed as previously described (Dignam et al., 1983; Gregory et al., 2001; Kemppainen et al., 1992).

Chromatin immunoprecipitation

LNCaP cells were grown in RPMI supplemented with 10% charcoal-stripped serum for four days before each experiment. Cells were treated with vehicle or PKI-166 for 10 min prior to addition of R1881 at the indicated concentrations. After 60 min of stimulation with R1881, crosslinking and further processing was performed as previously published (Louie et al., 2003). Immunoprecipitated DNA was amplified by PCR for 25 cycles. To monitor the linearity of the amplification conditions, serial dilutions of control DNA were amplified together with the experimental samples.

RNA-interference

Sequences for interfering RNAs (siRNA and shRNA) were 5′-CTACTTGGAG GACCGTCGC-3′ (EGFR RNAi #1), 5′-GTGTGTAACGGAATAGGTA (EGFR RNAi #2), 5′-GATCTTTGGGAGCCTGGCA-3′ (HER2 RNAi #1), and 5-ATCT TAGACGAAGCATACG-3′ (HER2 RNAi #2). siRNAs were obtained from Dharmacon, and hairpin RNAi constructs targeting HER2 were generated using the Clonetech pSIREN Kit.

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