**HER-2/neu Promotes Androgen-independent Survival and Growth of Prostate Cancer Cells through the Akt Pathway**

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**Abstract**

HER-2/neu has been implicated in the activation of androgen receptor (AR) and in inducing hormone-independent prostate cancer growth. Here we report that HER-2/neu activates Akt (protein kinase B) to promote prostate cancer cell survival and growth in the absence of androgen. Blocking of the Akt pathway by a dominant-negative Akt or an inhibitor LY294002 abrogates the HER-2/neu-induced AR signaling and cell survival/growth effects in the absence or presence of androgen. Akt specifically binds to AR and phosphorylates serines 213 and 791 of AR. Thus, Akt is a novel activator of AR required for HER-2/neu signaling to androgen-independent survival and growth of prostate cancer cells.

**Introduction**

Androgen plays a critical role in controlling the growth and survival of prostate cancer cells, and androgen ablation therapy usually achieves significant clinical responses in the beginning. Under the selective pressure of androgen withdrawal, however, prostate cancers progress to an androgen-independent stage (1). The mechanism for this progression to androgen independence is not completely understood. Although androgen-independent progression has been correlated with mutation of the AR (2, 3), most androgen-independent prostate cancer cells express AR and the androgen-dependent gene PSA, implying that these cells maintain a functional AR signaling pathway. Furthermore, it has been shown that MAP kinases are involved in activation of AR signal transduction (4, 5), suggesting that reactivation of the AR pathway by a hormone-independent mechanism may lead to androgen-independent prostate cancers. Recently, overexpression of HER-2/neu has been implicated in the activation of AR and in inducing hormone-independent prostate cancer growth (5, 6). HER-2/neu, a 185,000 transmembrane receptor tyrosine kinase with homology to members of the EGF receptor family, is overexpressed in ~30% of human breast and ovarian cancers (7). Unlike the other members of EGF receptors, HER-2/neu has an intrinsic tyrosine kinase activity that activates the receptor-mediated signal transduction in the absence of ligand. Although EGF binds to an EGF receptor to induce receptor dimerization and activate PI3K (8), HER-2/neu homodimer constitutively activates the PI3K-Akt pathway without extracellular stimulation (9). Activation of PI3K generates phosphatidylinositol-3,4,5-triphosphate, which in turn binds to the pleckstrin homology domain of serine/threonine kinase Akt, resulting in recruitment of Akt to the cell membrane. A conformational change of Akt follows, which enables residues Thr-308 and Ser-473 to be phosphorylated by upstream kinases, PDK-1 and PDK-2 or ILK, respectively (10). Activated Akt phosphorylates specific targets such as Bad (11), pro-caspase-9 (12), and transcription factor FKHLR1 (13) at the Akt phosphorylation consensus sequence R-X-R-X-S/T, thus promoting cell survival and blocking apoptosis. In this way, the PI3K-Akt pathway plays a critical role in antiapoptosis that may contribute to the pathogenesis of cancer (10). In the present study, we examined whether Akt and HER-2/neu are involved in the AR signaling pathway and whether they play a role in androgen-independent survival or growth of prostate cancer cells. We show that HER-2/neu activated Akt to promote prostate cancer cell survival and growth in the absence of androgen. The HER-2/neu-induced AR signaling and cell survival/growth effects were blocked by the DN-Akt or an inhibitor LY294002. Moreover, Akt specifically binds to AR and phosphorylates Ser-213 and Ser-791 of AR. Thus, our findings provide a molecular mechanism for the HER-2/neu-induced androgen-independent survival and growth of prostate cancer cells.

**Materials and Methods**

**Reporter and Cell Survival Assays.** LNCaP cells were plated the day before transfection at a density of 2 × 10^4 cells/well in six-well plates. The cells were cotransfected with a luc reporter plasmid (0.3 µg of PSA-luc or PRE-luc) and a β-gal expression plasmid (0.2 µg of CMV-β-gal) and expression plasmids or an empty vector (0.9 µg each) as indicated using liposomes. After transfection, the cells were cultured in phenol red-free medium supplemented with 5% of the c-FBS in the absence or presence of the synthetic androgen R1881 (NEN; 0.1 or 1.0 nM). Cell lysates were collected 48 h after transfection, and the luc activity of each sample was measured with the luciferase assay kit (Promega). β-gal activity was determined to normalize variations in transfection efficiency. The PSA-luc reporter construct (PSA-luc) was generated by subcloning a genomic DNA (~1.5 kb) containing the PSA promoter (640-bp) and enhancer (~820-bp) into the luc expression vector. The PRE-luc reporter (PRE-luc) contains two copies of the progesterone/androgen response element (14). For survival assays, LNCaP cells were prepared as described above and cotransfected with 0.2 µg of CMV-luc plasmid and 2 µg of Each expression plasmid as indicated. The transfected cells were cultured in the absence or presence of androgen as described above. The relative survival rate (percentage of cell survival) between the cultures in the absence and presence of androgen was determined by the luc activities and shown as a ratio, using the activity in the medium containing androgen as 100%. The data represent the mean value of at least three independent experiments, and statistical significance was calculated with the χ² test using SPSS software. *P < 0.05 was set as the criterion for statistical significance.

**Cell Proliferation Assays.** LNCaP cells were plated the day before transfection at 50% confluence in 100-mm dishes. The cells were cotransfected with

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2 The abbreviations used are: AR, androgen receptor; PSA, prostate-specific antigen; MAP, mitogen-activated protein; EGF, epidermal growth factor; PI3K, phosphatidylinositol-3-kinase; HA, hemagglutinin; CMV, cytomegalovirus; mAb, monoclonal antibody; β-gal, β-galactosidase; c-FBS, charcoal-treated FBS; BrdUrd, bromodeoxyuridine; wt, wild type; mut, mutant; GST, glutathione S-transferase; RFP, red fluorescent protein; luc, luciferase; DN-Akt, dominant-negative Akt; CA-Akt, constitutively active Akt.

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1.0 µg of pDsRed1-C1 plasmid (Clontech) and 15 µg of a constitutively active HER2/neu (HER2/neu), the Thr-1172 of which has been changed to Glu, or 15 µg of the control vector. After transfection, the cells were cultured in phenol red-free medium supplemented with 5% of c-FBS in the absence or presence of R1881 (0.1 nM). As controls, 36 h after transfection, the transfected cells were treated with 40 µM of LY294002 (Biomol), which is an inhibitor for the PI3K/Akt pathway. For BrdUrd labeling, 48 h after transfection, the cells were incubated with BrdUrd (10 µM) for 1 h. The transfected cells expressing the red fluorescent protein were selected by fluorescence-activated cell sorter.

The BrdUrd-labeled cells were detected using a BrdUrd labeling and detection kit (Roche) and evaluated under a fluorescent microscope. The percentages of BrdUrd-labeled cells were calculated based on ~400–800 RFP* cells from each sample.

**Immunoprecipitation, Western Blot, and Immunocomplex Kinase Assays.** LNCaP cells were washed with PBS, lysed in ice-cold RIPA buffer containing protease inhibitors, and centrifuged at 14,000 × g for 10 min at 4°C. For immunoprecipitation, 1 mg of each supernatant (cell lysate) was incubated with an anti-AR mAb (Pharmingen) or mouse IgG (negative control) overnight at 4°C. Then, protein G-agarose (Roche) was added and incubated for 2 h at 4°C with rotation. The immunocomplex was recovered by centrifugation, washed four times with lysis buffer, dissolved in loading buffer, and subjected to SDS-PAGE. For Western blot, the protein samples were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween 20 and incubated with an anti-p-Akt (S-473) antibody (New England Biolabs) and then with horseradish peroxidase-conjugated secondary antibodies according to the manufacturer’s instructions. The immunoblots were visualized by an enhanced chemiluminescence kit (ECL; Amersham). Immunocomplex kinase assays were performed as described previously (15). Specifically, 293T cells were transfected with the HA-tagged CA-Akt expression plasmid, the cells were harvested, and whole-cell lysates were prepared 48 h after transfection. After immunoprecipitation with an anti-HA mAb, phosphorylation of AR was determined by an immunocomplex kinase assay, using wt or mut GST-AR-N or GST-AR-C or histone 2B (Roche) as substrates. The GST-AR-N and GST-AR-C plasmids were constructed by subcloning of the cDNA fragments containing the N- or COOH-terminal domain of AR (AR-N or AR-C) by the PCR technique. The mutant GST-AR-N and GST-AR-C were generated by site-directed mutagenesis using specific oligonucleotides and a QuickChange site-directed mutagenesis kit (Stratagene) to change the Ser-213 and Ser-791 residues to Ala-213 and Ala-791, respectively. The GST-fusion proteins were purified by affinity chromatography on glutathione-Sepharose 4B (Amersham).

**Pull-Down Assays.** 293T cells were transfected with the HA-tagged CA-Akt expression plasmid, the cells were harvested, and whole-cell lysates were prepared 48 h after transfection. Fifty µl of glutathione-Sepharose were incubated with 90 µg of GST-AR-N or GST-AR-C or GST protein for 3 h and washed with washing buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP40] three times. The 293T cell lysates were mixed with the GST-AR-N or GST-AR-C or GST conjugated glutathione-Sepharose, incubated overnight at 4°C, and washed three times with washing buffer. The proteins bound to the GST-AR-N or GST-AR-C or GST-conjugated glutathione-Sepharose were analyzed by Western blotting using an anti-HA mAb (12CA5).

**Results and Discussion.**

Because activation of the AR signaling pathway by an androgen-independent survival mechanism may promote survival of prostate cancer cells during androgen deprivation, we investigated whether Akt is involved in the AR pathway and plays a role in androgen-independent survival or growth of prostate cancer cells. The PSA promoter/enhancer contains high-affinity AR binding sites and is up-regulated by androgen (16, 17). To examine the effects of Akt on PSA transcriptional regulation, we cotransfected LNCaP cells with the PSA-luc reporter plus any one of the following: a CA-Akt, a DN-Akt, an empty vector, or a PTEN (18), which is a tumor suppressor phosphatase that inhibits the PI3K/Akt pathway. We then assayed for the luc activities. CA-Akt activated the PSA-luc reporter 5–7-fold in a dose-dependent manner, whereas DN-Akt suppressed the reporter ~2-fold (Fig. 1a), suggesting that Akt activates PSA transcription. To compare the transactivation activity of Akt in the absence and presence of the hormone, we carried out the same DNA transfection experiments as described above, except that the cells were cultured in phenol red-free medium supplemented with the charcoal-treated serum to allow precise control of the concentration of androgen. In the absence of androgen, CA-Akt stimulated the PSA-luc reporter ~5-fold (Fig. 1b, Lane 4 versus Lane 1), and DN-Akt inhibited the reporter ~2-fold (Fig. 1b, Lane 7 versus Lane 1), suggesting that Akt can activate the PSA promoter/enhancer in the absence of androgen. Consistent with this concept, PTEN, which is a well-known PI3K/Akt inhibitor, also blocked activation of the PSA promoter/enhancer (Fig. 1b, Lanes 10–12) in the absence or presence of androgen, further suggesting that the Akt pathway may be required for transactivation of the PSA promoter/enhancer. Moreover, the PSA transcriptional activity induced by CA-Akt in the absence of R1881 (Fig. 1b, Lane 4 versus Lane 1) is slightly stronger than that stimulated by R1881 alone (Fig. 1b, Lane 3 versus Lane 1); activation of PSA transcription appears to be less effective by R1881 in the presence of CA-Akt (~1.6-fold; Fig. 1b, Lane 6 versus Lane 4) than in the absence of
CA-Akt (~4-fold; Fig. 1b, Lane 3 versus Lane 1). Taken together, these results imply that Akt is involved in androgen-independent transactivation function.

To further examine whether the androgen-independent transactivation of Akt can be extended to cell survival of prostate cancer cells upon androgen withdrawal, we cotransfected LNCaP cells with the CMV-luc reporter plus CA-Akt or DN-Akt or an empty vector and cultured in the same medium in the absence or presence of R1881 (0.1 nm), with or without LY294002 (LY) as indicated. The normalized luc activity was indicated as described in the legend to Fig. 1. Bars, SD. a, LNCaP were cotransfected with the CMV-luc reporter and a CMV-β-gal vector and HER-2/neu* or HER-2/neu* plus DN-Akt or an empty vector and cultured in the same medium in the absence or presence of R1881 (0.1 nm). The percentage of cell survival represents the ratio of the normalized luc activities between the cells cultured without R1881 and with R1881. Bars, SD. b, LNCaP were cotransfected with pDsRed1-C1 encoding a RFP and HER-2/neu* or an empty vector and cultured in the same medium in the absence or presence of R1881 (0.1 nm), with or without LY as indicated. The transfected cells were incubated with BrdUrd, the RFP+ cells were sorted by fluorescence-activated cell sorter, and the sorted cells were plated onto the poly-L-lysine-coated slides using Cytoospin II. The BrdUrd-labeled cells were detected and evaluated under a fluorescence microscope. The percentage of BrdUrd-labeled cells were determined based on ~400–800 RFP+ cells from each sample; bars, SD. d, representative fluorescent pictures of the RFP+ cells are depicted.
activation of the AR pathway by HER-2/neu requires functional Akt. The PSA-luc reporter construct encodes 1400 bp of sequence consisting of a high-affinity androgen responsive element in the promoter and an androgen responsive region in an upstream enhancer (16, 17). To exclude the possibility that some other promoter or enhancer elements may contribute to PSA activation by androgen, we further analyzed the PRE-luc reporter, which contains two copies of high-affinity androgen response element (14). Similarly, HER-2/neu activated the PRE-luc reporter significantly in the presence or absence of anti-HA mAb. Specific IgG and GST were used as a negative control.

To this end, we cotransfected LNCaP cells with the CMV-luc reporter and HER-2/neu expression plasmids. The reporter construct encodes 1400 bp of sequence consisting of the high-affinity androgen responsive element in the promoter and an androgen responsive region in an upstream enhancer (16, 17). The PSA-luc reporter construct encodes 1400 bp of sequence consisting of the high-affinity androgen responsive element in the promoter and an androgen responsive region in an upstream enhancer (16, 17). The PSA-luc reporter construct encodes 1400 bp of sequence consisting of the high-affinity androgen responsive element in the promoter and an androgen responsive region in an upstream enhancer (16, 17). The PSA-luc reporter construct encodes 1400 bp of sequence consisting of the high-affinity androgen responsive element in the promoter and an androgen responsive region in an upstream enhancer (16, 17). The PSA-luc reporter construct encodes 1400 bp of sequence consisting of the high-affinity androgen responsive element in the promoter and an androgen responsive region in an upstream enhancer (16, 17). The PSA-luc reporter construct encodes 1400 bp of sequence consisting of the high-affinity androgen responsive element in the promoter and an androgen responsive region in an upstream enhancer (16, 17). The PSA-luc reporter construct encodes 1400 bp of sequence consisting of the high-affinity androgen responsive element in the promoter and an androgen responsive region in an upstream enhancer (16, 17). The PSA-luc reporter construct encodes 1400 bp of sequence consisting of the high-affinity androgen responsive element in the promoter and an androgen responsive region in an upstream enhancer (16, 17). The PSA-luc reporter construct encodes 1400 bp of sequence consisting of the high-affinity androgen responsive element in the promoter and an androgen responsive region in an upstream enhancer (16, 17). The PSA-luc reporter construct encodes 1400 bp of sequence consisting of the high-affinity androgen responsive element in the promoter and an androgen responsive region in an upstream enhancer (16, 17). The PSA-luc reporter construct encodes 1400 bp of sequence consisting of the high-affinity androgen responsive element in the promoter and an androgen responsive region in an upstream enhancer (16, 17). The PSA-luc reporter construct encodes 1400 bp of sequence consisting of the high-affinity androgen responsive element in the promoter and an androgen responsive region in an upstream enhancer (16, 17).

Fig. 3. Activated Akt is associated with AR in vivo and in vitro and specifically phosphorylates Ser-213 and Ser-791 of AR in vitro. a, the endogenous AR in LNCaP cell lysate was immunoprecipitated with an anti-AR mAb or a control mouse IgG and then analyzed by Western blot using an anti-p-Akt antibody. b, two consensus Akt phosphorylation sites (RXRXXS/T) are conserved among human, rat, and mouse AR amino acid sequences. The Ser-213 and Ser-791 residues are located within the NH$_2$-terminal half and the COOH-terminal domain of human AR, respectively. c, pull-down (in vitro) assays of HA-CA-Akt with GST-AR-N or GST-AR-C. 293T cells were transfected with the HA-CA-Akt expression plasmid, the cells were harvested, and whole-cell lysates were prepared 48 h after transfection. After immunoprecipitation with an anti-HA mAb, phosphorylation of AR was determined by an immunocomplex kinase assay using wt or mut GST-AR-N and GST-AR-C as substrates. As a control for substrates, an equal amount of each GST-fusion protein was subjected to SDS-PAGE (10%), transferred to a nitrocellulose membrane, and analyzed by Western blot using an anti-GST antibody (bottom panel). e, as a kinase control for Akt, the same immunocomplex kinase assay was performed as described above using histone 2B as a substrate.
CA-Akt. Whole-cell lysates of the transfected cells were immunoprecipitated using an anti-HA mAb, and the immunoprecipitated proteins were subjected to immunocomplex kinase assays using GST-AR-N (wt or mut) and GST-AR-C (wt or mut) as substrates. Phosphorylation of GST-AR-N/wt and GST-AR-C/wt was readily detected, whereas phosphorylation of GST-AR-N/mut and GST-AR-C/mut was not detectable (Fig. 3d), suggesting that Ser-213 and Ser-791 were specifically phosphorylated by CA-Akt. As a positive control, the same immunoprecipitated proteins were subjected to an immunocomplex kinase assay using histone 2B as a substrate (Fig. 3e). As shown in Fig. 4, a model is depicted to illustrate the proposed parallel cell survival and proliferation pathways induced by HER-2/neu via the Akt pathway from the current study or the MAP kinase pathway as well reported previously (4, 5). To our knowledge, this is the first evidence that HER-2/neu activates AR via the Akt pathway and may provide an interpretation of how HER-2/neu can restore AR signaling to prostate cancer cells during androgen ablation. The effect of Ser-213 and Ser-791 mutants on AR function and response to HER-2/neu-Akt activation deserves further investigation and may open an alternative avenue for understanding the molecular mechanism of the androgen-independent survival and growth of prostate cancer cells. It may lead to a new direction for developing novel anticancer therapies for the androgen-refractory prostate cancers.

References


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