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 gene (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 M App 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 modimer 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), procaspase-9 (12), and transcription factor FKHL1 (13) at the Akt phosphorylation consensus sequence R-X-R-X-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 x 10^5 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 an 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 luc 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 (percent 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 χ^2 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...
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 and plated onto poly-l-lysine-coated slides using Cytospin II. 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 x g for 10 min at 4°C. For immunoprecipitation, 1 μg 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, and 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

![Fig. 1](image-url)

**Fig. 1. Akt activates the PSA promoter/enhancer and promotes survival of androgen-independent prostate cancer cells upon androgen deprivation.** a. androgen-dependent LNCaP cells were cotransfected with the PSA-luc reporter and a CMV-β-gal vector plus a constitutively active Akt (CA-Akt) or a dominant-negative Akt (DN-Akt) or an empty vector as indicated and cultured in RPMI 1640 supplemented with 5% FBS. +, double dose of +, which is 0.6 μg of DNA. The luc activity was measured after 48 h and expressed as an arbitrary unit; the β-gal activity was measured to normalize variations in transfection efficiency. b. LNCaP cells were cotransfected with the PSA-luc reporter and a CMV-β-gal vector plus CA-Akt or DN-Akt or PTEN or an empty vector and cultured in phenol red-free medium supplemented with 5% of the c-FBS in the absence or presence of the synthetic androgen R1881 (0.1 or 1.0 nM) as indicated. The normalized luc activity was indicated as described above. c. LNCaP cells were cotransfected with the CMV-luc reporter and a CMV-β-gal vector plus CA-Akt or DN-Akt or PTEN or an empty vector and cultured in RPMI 1640 supplemented with 5% of either c-FBS or FBS. The percentage of cell survival represents the ratio of the normalized luc activities between the cells cultured in c-FBS and those cultured in FBS. d. LNCaP cells were cotransfected with the vectors as described above and cultured in phenol red-free medium supplemented with 5% c-FBS in the absence or presence of R1881 (0.1 mM). The percentage of cell survival indicates the ratio of the normalized luc activities between the cells cultured without R1881 and with R1881. The data represent the mean value of at least three independent experiments; bars, SD.
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 phenol red-free medium supplemented with 5% FBS in the absence or presence of R1881 (0.1 or 1.0 nM), with or without LY294002 (LY) as indicated. The normalized luc activity was indicated as described in the legend to Fig. 1. Bars, SD. b, 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. c, 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<sup>+</sup> cells were sorted by fluorescence-activated cell sorter, and the sorted cells were plated onto the poly-L-lysine-coated slides using Cytospin 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<sup>+</sup> cells from each sample; bars, SD. d, representative fluorescent pictures of the RFP<sup>+</sup> cells are depicted.

Because HER-2/neu has been shown recently to activate AR signaling (9, 10) and we have demonstrated previously that HER-2/neu activates the Akt pathway, we determined whether Akt may be involved in the HER-2/neu-activated AR signaling. We cotransfected LNCaP cells with the PSA-luc reporter and an activated form of HER-2/neu (HER-2/neu*) or HER-2/neu* plus DN-Akt and cultured the cells in the hormone-free medium as described above. HER-2/neu* activated the PSA promoter/enhancer ~8-fold (Fig. 2a, Lane 5 versus Lane 2, at 0.1 nM R1881) or ~5-fold (Fig. 2a, Lane 6 versus Lane 3, at 1.0 nM R1881) in the presence of R1881, and ~10-fold (Fig. 2a, Lane 4 versus Lane 1) in the absence of R1881. However, this stimulatory effect of HER-2/neu* was dramatically reduced by DN-Akt or an Akt inhibitor LY294002 (Fig. 2a), suggesting that
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 attribute 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 R1881. However, this stimulatory effect was blocked by DN-Akt (data not shown), confirming that HER-2/neu activates AR signaling via the Akt pathway.

To address the critical question of whether HER-2/neu can enhance prostate cancer cell survival upon androgen deprivation, we cotransfected LNCaP cells with the CMV-luc reporter and HER-2/neu* or HER-2/neu* plus DN-Akt or PTEN and cultured the cells in the hormone-free medium with or without R1881 as described above. Importantly, HER-2/neu* significantly increased survival (~30%) of LNCaP cells in the absence of R1881, whereas this enhancing effect by HER-2/neu* was abolished by DN-Akt (Fig. 2b). These results suggest that HER-2/neu promotes prostate cancer cell survival upon androgen withdrawal through the Akt pathway. Furthermore, to examine whether HER-2/neu can stimulate prostate cancer cell proliferation during androgen withdrawal, we measured the DNA synthesis rate by the BrdUrd incorporation rate. We cotransfected LNCaP cells with a red fluorescent protein expression vector pDsRed1-C1 and HER-2/neu* or an empty vector and cultured the cells in the hormone-free medium with or without R1881. In addition, one set of the HER-2/neu*-transfected cells were treated with LY294002. Subsequently, the red fluorescence-positive cells were sorted, plated on slides by cyto spin, and assayed for proliferation by BrdUrd incorporation. Significantly, HER-2/neu* augmented the BrdUrd incorporation rate (i.e., growth rate) of LNCaP cells ~2-fold (Lane 3 versus Lane 1) in the absence of R1881 and ~1.5-fold (Lane 4 versus Lane 2) in the presence of R1881 (Fig. 2c). Representative fluorescent pictures are shown (Fig. 2d). However, this growth-stimulatory effect of HER-2/neu* was strongly blocked by LY294002 (Fig. 2c), confirming that activation of the androgen-independent growth by HER-2/neu requires a functional PI3-K/Akt pathway. Taken together, HER-2/neu promotes prostate cancer cell survival and growth upon androgen deprivation via the Akt pathway.

Because Akt can activate the AR pathway, we investigated whether the interactions between Akt and AR can occur in vivo. Whole-cell lysates of LNCaP cells were immunoprecipitated using an anti-AR mAb or mouse IgG, and the immunoprecipitated proteins were subjected to Western blotting using an antibody against phosphorylated Akt (p-Akt). Our results showed that p-Akt (activated Akt) was coimmunoprecipitated with an mAb against AR but not with the control IgG (Fig. 3a), suggesting that the endogenous AR is specifically associated with activated Akt in vivo. Comparison of the amino acid sequences of AR among human, rat, and mouse reveals two conserved putative consensus Akt phosphorylation sites, Ser-213 and Ser-791, based on the human sequence (Fig. 3b). Therefore, we examined which sites of the AR protein can interact with CA-Akt in vitro. We generated and purified GST-fusion proteins containing the NH2-terminal half of AR (designated GST-AR-N) and the COOH-terminal portion of AR (designated GST-AR-C), where Ser-213 and Ser-791 residues reside in AR-N and AR-C, respectively. Whole-cell lysates of HA-CA-Akt transfected 293T cells were incubated with glutathione-Sepharose preloaded with either GST-AR-N or GST-AR-C or GST protein. The bound proteins were eluted and immunoblotted with an anti-HA mAb. Specific M, 62,000 HA-AR proteins were detected in the GST-AR-N- and GST-AR-C-bound fractions as well as in the total lysates (positive control) but not in the GST (negative control) bound fractions (Fig. 3c). These results suggest that both domains (Akt phosphorylation sites) of the AR protein are specifically associated with CA-Akt in vitro. Furthermore, we determined whether these two Akt target sites can be phosphorylated by CA-Akt, using immunocomplex kinase assays. To test the specificities of Akt phosphorylation, these two Ser-213 and Ser-791 residues within GST-AR-N and GST-AR-C were mutated to Ala residues, designated GST-AR-N/mut and GST-AR-C/mut, respectively. To produce CA-Akt, 293T cells were transiently transfected with HA-
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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