Up-regulation of Akt3 in Estrogen Receptor-deficient Breast Cancers and Androgen-independent Prostate Cancer Lines*

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We measured the insulin-stimulated amount of Akt1, Akt2, and Akt3 enzymatic activities in four breast cancer cell lines and three prostate cancer cell lines. In the estrogen receptor-deficient breast cancer cells and the androgen-insensitive prostate cells, the amount of Akt3 enzymatic activity was approximately 20–60-fold higher than in the cells that were estrogen- or androgen-responsive. In contrast, the levels of Akt1 and -2 were not increased in these cells. The increase in Akt3 enzyme activity correlated with an increase in both Akt3 mRNA and protein. In a prostate cancer cell line lacking the tumor suppressor PTEN (a lipid and protein phosphatase), the basal enzymatic activity of Akt3 was constitutively elevated and represented the major active Akt in these cells. Finally, reverse transcription-PCR was used to examine the Akt3 expression in 27 primary breast carcinomas. The expression levels of Akt3 were significantly higher in the estrogen receptor-negative tumors in comparison to the estrogen receptor-positive tumors. To see if the increase in Akt3 could be due to chromosomal abnormalities, the Akt3 gene was assigned to human chromosome 1q44 by fluorescence in situ hybridization and radiation hybrid cell panel analyses. These results indicate that Akt3 may contribute to the more aggressive clinical phenotype of the estrogen receptor-negative breast cancers and androgen-insensitive prostate carcinomas.

Akt (also called protein kinase B) is a serine/threonine protein kinase that has been implicated in mediating a variety of biological responses including inhibiting apoptosis and stimulating cellular growth (reviewed in Ref. 1). There are three mammalian isoforms of this enzyme, Akt1, Akt2, and Akt3 (1). Akt1 was found to be the cellular homolog of a viral oncogene (v-Akt) that causes leukemia in mice (2). Further confirming the oncogenic potential of Akt, Akt1 was found to be overexpressed in 20% of gastric adenocarcinomas, and Akt2 was overexpressed in 3% of breast cancers, 15% of ovarian cancers, and 12% of pancreatic cancers because of gene amplification (2–4).

Moreover, recent studies have documented that the tumor suppressor called PTEN or MMAC1 is actually a lipid phosphatase that can dephosphorylate phosphatidylinositol 3,4,5-trisphosphate (5). Since this lipid is one of the primary activators of Akt (1), loss of PTEN results in a high basal activity of Akt in a variety of tumors including glioblastomas and prostate cancer lines, while reintroduction of PTEN suppresses Akt activity (6–12). Finally, much of the ability of PTEN to regulate the cell cycle and induce apoptosis appears to be mediated via its ability to regulate Akt enzymatic activity (6–12).

Although the cDNA encoding rat Akt3 was identified over 3 years ago (13), little information has been reported on this isoform. Like Akt1 and Akt2, Akt3 contains a pleckstrin homology domain, which is highly homologous to those of Akt1 and -2 and presumably binds phosphatidylinositol 3,4,5-trisphosphate and is involved in activation of this enzyme (13). Also, like these other isoforms, Akt3 contains a threonine residue in the same region as a critical regulatory phosphorylation site present in the activation loop of Akt1 and -2 and is phosphorylated by the same enzyme, PDK1, that phosphorylates this site in the other isoforms (14). Unlike Akt1 and -2, Akt3 was initially reported to lack the second critical regulatory phosphorylation site in its carboxy tail (13), although this has now been questioned (15, 16). In addition, its tissue distribution appears to be more limited than that of Akt1 and -2, being primarily expressed in brain and testis (13).

In the present studies, we have examined the levels of Akt3 in both breast cancer and prostate cancer cell lines. We find that both the Akt3 enzymatic activity and mRNA are elevated in breast cancer cell lines and tumors that lack the estrogen receptor (ER)1 as well as in prostate cancer cell lines that are androgen-insensitive. These results indicate that Akt3 may contribute to the more aggressive clinical phenotype of these hormone-unresponsive breast and prostate carcinomas (17–20).

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and the RT-PCR kit were from Life Technologies, Inc. Total RNA and poly(A) RNA were isolated using the RNeasy kit from Qiagen (Chatsworth, CA) and the Fast Track 2.0 kit from Invitrogen (Carlsbad, CA), respectively. The primers for the Akt1, Akt2, Akt3, ER, PTEN/MMAC1, and β-actin were from Operon Technologies Inc. (Alameda, CA). [γ-32P]ATP (3000 Ci/mmol) was from NEN Life Science Products, and [α-32P]dCTP (3000 Ci/mmol) was from Amersham Pharmacia Biotech. The Akt substrate peptide (21) was synthesized in the Beckman-PAN facility (Stanford, CA). Nitrocellulose (Protran) and nylon (Nytran) membranes were from Schleicher &

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1 The abbreviations used are: ER, estrogen receptor; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; FISH, fluorescence in situ hybridization; HA, hemagglutinin.
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Schuell; the random priming kit, anti-HA monoclonal antibody (12C5), and FuGene6 transfection reagent were from Roche Molecular Biochemicals; and anti-FLAG antibodies (M5) were from Sigma. A monoclonal antibody to the ER antibody (HC-20) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibodies against Akt3 and Akt2 antibodies directed against the catalytic and pleckstrin homology domains were produced as described (22). Anti-Akt2 antibodies were a gift of Dr. Birnbaum (23). A monoclonal anti-Akt1 antibody and a sheep anti-Akt3 antibody were from Transduction Laboratories (Lexington, KY) and Upstate Biotechnology, Inc. (Lake Placid, NY), respectively. Protein A-Sepharose and protein G-Sepharose were from Promega (Madison, WI) and Amersham Pharmacia Biotech, respectively.

Expression Plasmids—The pECE construct coding the HA-tagged human Akt1 was as described (24). The pECE constructs encoding FLAG-tagged rat Akt2 and FLAG-tagged rat Akt3 (13) were kindly provided by Dr. Kikkawa (Kobe, Japan).

Transient Transfections—Human embryonic kidney 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37 °C, in an atmosphere containing 5% CO2. Cells were transfected using FuGene6 with 1 μg of plasmid DNA (HA-tagged Akt1-pECE, FLAG-tagged Akt2-pECE, FLAG-tagged Akt3-pECE, or vector without cDNA insert). Transfected 293T cells were extracted in 1 ml of lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml bacitracin, and 1 mM sodium orthovanadate in 1 ml of lysis buffer (26). Akt1, Akt2, and Akt3 were detected by Western blotting with anti-Akt antibodies (1:1000), while Akt1 was detected with anti-HA antibodies (1:5000).

Akt Enzyme Assays—Breast cancer cells (MCF-7, T-47D, MDA-MB-231, HBL-100) and prostate cancer cells (LNCaP, DU-145, PC-3) were maintained in a humidified atmosphere of 5% CO2 in RPMI 1640 medium supplemented with 5% fetal calf serum, 3% l-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin. Cells were serum-starved overnight, stimulated with insulin, lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (w/v) Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM/ml bacitracin, 1 mM Na3VO4, 30 mM NaPPi, 10 mM NaF, 100 mM okadaic acid). Following centrifugation as above, the supernatants were incubated for 2 h at 4 °C with protein A-Sepharose beads coated with 2.5 μl of anti-Akt1, 5.0 μl of anti-Akt2, or 2.5 μl of anti-Akt3 antibodies. Immunoprecipitates were washed three times with the lysis buffer and twice with the kinase assay buffer (50 mM Tris-HCl, pH 7.5, 10 μM MgCl2, 1 μM dithiothreitol) and assayed using GSK-3 peptide (GRPPRTSSFAEG) as substrate as described (25). Following the kinase reaction, the phosphorylated peptide was separated from unincorporated [gamma-32P]ATP on a 40% polyacrylamide gel and transferred to nitrocellulose membranes. Akt3 and Akt2 were detected by Western blotting with anti-Akt3 antibodies (1:1000), while Akt1 was detected with anti-HA antibodies (1:5000).

Reverse Transcription-PCR—Total RNA was extracted from tissue culture cells using RNAeasy (Qiagen). Primary human breast tumor specimens were processed using Trizol reagent (Life Technologies). The quality and quantity of the RNA were determined by measuring the absorbance at 260 nm. For RT-PCR studies, first strand and cDNA were generated from 1–5 μg of total RNA with reverse oligo(dT) primer and SuperScript II reverse transcriptase (Life Technologies). Primers for PCR of human pETCE, or vector without cDNA insert). Transfected 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum at 37 °C, in an atmosphere containing 5% CO2. Cells were transfected using FuGene6 with 1 μg of plasmid DNA (HA-tagged Akt1-pECE, FLAG-tagged Akt2-pECE, FLAG-tagged Akt3-pECE, or vector without cDNA insert). Transfected 293T cells were extracted in 1 ml of lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM/ml bacitracin, 1 mM Na3VO4, 30 mM NaPPi, 10 mM NaF, 100 mM okadaic acid). Following centrifugation as above, the supernatants were incubated for 2 h at 4 °C with protein A-Sepharose beads coated with 2.5 μl of anti-Akt1, 5.0 μl of anti-Akt2, or 2.5 μl of anti-Akt3 antibodies. Immunoprecipitates were washed three times with the lysis buffer and twice with the kinase assay buffer (50 mM Tris-HCl, pH 7.5, 10 μM MgCl2, 1 μM dithiothreitol) and assayed using GSK-3 peptide (GRPPRTSSFAEG) as substrate as described (25). Following the kinase reaction, the phosphorylated peptide was separated from unincorporated [gamma-32P]ATP on a 40% polyacrylamide gel and transferred to nitrocellulose membranes. Akt3 and Akt2 were detected by Western blotting with anti-Akt3 antibodies (1:1000), while Akt1 was detected with anti-HA antibodies (1:5000).

Western Blot Analyses—Breast cancer cells and prostate cancer cells were homogenized with lysis buffer (as described above). After centrifugation at 15,000 × g for 30 min, the supernatants (1 mg of protein) were incubated for 2 h at 4 °C with either protein G-Sepharose beads coated with 5 μg of sheep anti-Akt1 antibodies or protein A-Sepharose beads coated with rabbit anti-Akt1 antibodies. The bound proteins were resolved on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Akt3 and Akt2 were detected by Western blotting with the rabbit anti-Akt3 antibodies (1:1000), while Akt1 was detected with the monoclonal anti-Akt1 antibody (1:1000).

Reverse Transcription-PCR—Total RNA was extracted from tissue culture cells using RNAeasy (Qiagen). Primary human breast tumor tissues were collected fresh from mastectomy and biopsy specimens and snap frozen in liquid nitrogen (26). Total RNA was isolated from these specimens using Trizol reagent (Life Technologies). The quality and quantity of the RNA were determined by measuring the absorbance at 260 nm. For RT-PCR studies, first strand and cDNA were generated from 1–5 μg of total RNA with reverse oligo(dT) primer and SuperScript II reverse transcriptase (Life Technologies). Primers for PCR of

RESULTS

Akt1, -2,-3 Activities in Breast and Prostate Cancer Cells—To measure the amount of Akt1, -2, and -3 in cells, we utilized distinct antibodies for each isoform (see “Experimental Procedures”). To test the specificity of these antibodies, we first examined them for their ability to precipitate epitope-tagged expressed forms of these enzymes. Each antibody was found to preferentially precipitate its intended target, demonstrating their relative specificity (Fig. 1A). These antibodies were then utilized to examine the levels of the three isoforms of Akt in four breast cancer cell lines, two which express the ER (MCF-7, T-47D) and two which do not express the ER (MDA-MB-231, HBL-100). Cells were treated with buffer or insulin (to maximally stimulate the Akt enzymatic activity) and lysed, and the lysates were precipitated with antibodies to the three isoforms. An insulin-stimulated Akt1 activity was present in all four cell lines (Fig. 1B). Almost no detectable Akt2 activity was measured with or without insulin in all four cell lines (data not shown). Akt3 activity was 30–80-fold higher in the two ER-negative cell lines than in the two ER-positive cell lines (Fig. 1B).

We then measured the activity of the Akt isoforms in three prostate cancer cell lines, one of which is androgen-sensitive (LNCaP) and two of which are androgen-insensitive (DU-145 and PC-3). Detectable Akt1 activity was present in all three cell lines (Fig. 1C), while little Akt2 activity was found (data not shown). Akt3 activity was 20–40-fold greater in the two androgen-insensitive cells than in the androgen-sensitive cells (Fig. 1C). PC-3, which does not contain PTEN (Ref. 27; confirmed with the cells used in the present work), exhibited a 40–100-fold elevated basal level of Akt3 activity in comparison with the
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**Fig. 1.** Levels of Akt1, Akt2, and Akt3 enzymatic activities in breast cancer and prostate cancer cell lines. A, specificity of the antibodies. Human embryonic kidney 293T cells were transiently transfected with plasmids encoding either HA-tagged Akt1, FLAG-tagged Akt2, or FLAG-tagged Akt3. Cells were lysed, and the lysates were precipitated with antibodies to Akt1, -2, or -3. The precipitates were washed and analyzed by immunoblotting with antibodies to either the HA tag or the FLAG tag. B, Akt enzymatic activities in breast cancer cell lines. The indicated cells were serum-starved overnight, treated with or without 100 nM insulin for 10 min, and lysed, and the lysates were immunoprecipitated with the indicated antibodies. The immunoprecipitates were assayed for Akt enzymatic activity as described under "Experimental Procedures." Results shown are representative from three experiments. The values for Akt2 enzymatic activity were not significantly above the control Ig values. C, Akt enzymatic activities in prostate cancer cell lines. The indicated cells were serum-starved overnight, treated with or without 100 nM insulin for 10 min, and lysed, and the lysates were immunoprecipitated with the indicated antibodies. The immunoprecipitates were assayed for Akt enzymatic activity as described under "Experimental Procedures." Results shown are representative from three experiments.

DU-145 cells. LNCaP, which also does not have a functional PTEN, exhibited a constitutively active Akt1.

**Measurement of Akt3 Protein Levels in Breast Cancer Cells and Prostate Cancer Cells**—To determine whether the Akt3 enzymatic activities measured above reflected the levels of the Akt3 protein present in these different cells, we utilized a commercially available sheep antibody to Akt3 to precipitate the protein and then Western blotted these precipitates with the rabbit polyclonal antibody to Akt3. As controls, the levels of Akt1 protein and the ER in these cells were also monitored by immunoprecipitation and Western blotting. The two ER-negative cells (MDA-MB-231, HBL-100) both contained an Akt3 band, whereas the two ER-positive cells (MCF-7, T-47D) did not contain a detectable Akt3 band (Fig. 2A). In contrast, all four cell lines contained an Akt1 band.

Similar studies were then performed on the prostate cancer cell lines. The two androgen-insensitive cell lines (PC-3 and DU-145) were both found to contain Akt3, whereas no detectable Akt3 band was observed in the androgen-sensitive cells, LNCaP (Fig. 2B). Again, all three cell lines contained an Akt1 band.

**Measurement of Akt1 and Akt3 mRNA Levels in Breast and Prostate Cancer Cells and Breast Tumor Samples**—To determine whether the increase in Akt3 enzyme activity and protein was due to an increase in Akt3 mRNA, Northern blot analyses was performed. Akt3 mRNA was found to be greatly elevated in two ER-negative breast cancer cell lines (MDA-MB-231, HBL-100) and in a breast cancer cell line (BT-20) that exhibits very low levels of ER expression in comparison with the five ER-positive cells tested (Fig. 3). Akt3 transcripts of 7.7, 5.3, and 1.4 kilobase pairs were observed. In contrast, no specific pattern was observed with Akt1 mRNA levels, with the greatest amounts being present in MCF-7, MDA-MB-361, BT-474, and HBL-100 (Fig. 3).

To assess Akt3 levels in primary human breast tumor tissues, semiquantitative RT-PCR was utilized. Controls first verified that this method also demonstrated elevated levels of the Akt3 mRNA in the two breast cancer cell lines that are ER-negative (the MDA-MB-231 and HBL-100 cells) (Fig. 2A). Using this method, we also found elevated levels of Akt3 mRNA in the two androgen-insensitive prostate cancer cell lines (DU-145 and PC-3) in comparison with the androgen-sensitive cells (Fig. 2B). Twenty-seven primary breast carcinomas were then analyzed. Concurrently, the levels of Akt1, Akt2, Akt3, β-actin, and ER mRNA were also measured (Fig. 4). There was a significant correlation between a low level of ER mRNA and elevated Akt3 mRNA (p = 0.04) in these samples, although some samples with ER did contain Akt3 (Fig. 4A). In contrast, there was no significant correlation between the ER mRNA levels and Akt1 mRNA (p = 0.69) (Fig. 4B) or Akt2 mRNA (p = 0.58) (data not shown). By RT-PCR, human mammary epithelial cells had high levels of Akt3 and low levels of ER (data not shown).

**Chromosomal Localization of the Human Akt3 Gene**—Since many oncogenes are activated by chromosomal rearrangements and/or gene amplifications, we determined the chromosomal localization of the Akt3 gene. We first used radiation hybrid mapping. The GeneBridge 4 Radiation Hybrid Panel
(Research Genetics) was screened by PCR using primers directed to the 3'-untranslated region, which gave a product with human genomic DNA but not with Chinese hamster DNA. PCR product was identified with the DNA of 22 of the 93 RH cell lines, and Akt3 was placed on chromosome 1, 5.45 cR from D1S2842 and 20.8 cR from AFM155XC11. These results indicated that the human Akt3 gene is localized to human chromosome 1q44. To verify these results, a human genomic clone for Akt3 was utilized for FISH (Fig. 5). Of 80 checked metaphase cells, 75 showed that the Akt3 gene was localized to the terminus of the long arm of chromosome 1, in a position corresponding to band 1q44. Thus, the results obtained by in situ hybridization and radiation hybrid panel were in accord.

**DISCUSSION**

A wide variety of studies have implicated the serine/threonine kinase called Akt in the transformation of cells (1–4, 28). This kinase can both inhibit apoptosis in cells as well as stimulate their growth (reviewed in Ref. 1). A mutant form of this enzyme was found to be the transforming component of AKT8 retrovirus (2). In addition, a tumor suppressor, called PTEN or MMAC1, regulates the basal activity of this enzyme and induces cell death, which is rescued by constitutively active forms of the enzyme (5–12). Since three isoforms of Akt have been identified, it is not clear which of these isoforms is involved (1).

In several cancers, an increase in Akt2 protein and mRNA was observed due to gene amplification (2–4).

In the present studies, we have examined the levels of the three Akt isoforms in various breast cancer lines as well as prostate cancer cells. In all four breast cancer cell lines, Akt1 was present and stimulated by insulin treatment. In contrast, very little Akt2 enzymatic activity was detected. Most importantly, Akt3 enzymatic activity was 30–60-fold higher in the two ER-negative cell lines (MDA-MB-231 and HBL-100) than in the two ER-positive cell lines (MCF-7 and T-47D). Similarly, Akt3 enzymatic activity was 20–40-fold higher in two andro-
gen-insensitive prostate cancer cell lines (DU-145 and PC-3) in comparison with an androgen-sensitive prostate cancer cell line (LNCaP). These findings indicate that the increases in Akt3 isoform may be more generally true for other cancers as well. In PC-3, a line of cells that lacks the lipid phosphatase PTEN, a markedly elevated basal level of Akt enzymatic activity was observed. This basal activity was predominantly contributed by Akt3. These results indicate that in some tumors that lack PTEN, the increase in basal activity of Akt may be primarily due to Akt3.

The higher level of Akt3 enzymatic activity in both the ER-negative breast cancer cells and the androgen-insensitive prostate cancer cells correlates with an increased expression of Akt3 protein and mRNA. The increase in Akt3 mRNA in these cell lines did not appear to be due to gene amplification in the cells tested (data not shown). Also, the chromosomal region identified as containing the Akt3 gene, 1q44, has not been found to be amplified in different cancers (29). However, it is close to a region (1q42.2–43) that has been identified as predisposing individuals to early onset prostate cancer (30).

The inverse correlation between the ER levels and Akt3 levels observed in the breast cancer cell lines also appeared to persist in a panel of 27 primary breast carcinomas, although several samples did show a divergence from this pattern. It is possible that the divergence in some tumors may represent nonfunctional ER and/or tissue heterogeneity. It is also possible that the ER does not directly repress the expression of the Akt3 gene. In support of this latter hypothesis, attempts to express the ER in the ER-negative cells did not result in a decrease in the levels of Akt3 mRNA (data not shown), suggesting a more complex interaction. Alternatively, it is possible that since ER-negative breast carcinomas are generally less hormonally responsive than hormone-responsive tumors (17–20), it is possible that Akt3 contributes to this phenotype. For example, the ability of Akt to inhibit apoptosis induced by a wide variety of agents could make the tumors with high Akt3 levels more resistant to chemotherapeutic treatments. Furthermore, the ability of Akt to inhibit death of cells after detachment from the extracellular matrix could promote the frequency of metastasis (32).

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