Heregulin Induces Phosphorylation of BRCA1 through Phosphatidylinositol 3-Kinase/AKT in Breast Cancer Cells*

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The breast cancer susceptibility gene BRCA1 encodes a nuclear phosphoprotein that acts as a tumor suppressor. Phosphorylation of BRCA1 has been implicated in altering its function, however, the pathway(s) that leads to the phosphorylation of BRCA1 has not been described. Here, a signaling pathway by which heregulin induces cell cycle-independent phosphorylation of BRCA1 was delineated. We showed that heregulin stimulation induced the phosphorylation of BRCA1 and concomitant activation of the serine/threonine kinase AKT in T47D human breast cancer cells. Heregulin-induced phosphorylation of BRCA1 was abrogated by phosphatidylinositol 3-kinase (PI3K) inhibitors and by a dominant-negative AKT. In the absence of heregulin, the ectopic expression of the constitutively active p110 subunit of PI3K was sufficient to induce BRCA1 phosphorylation. Furthermore, the purified glutathione S-transferase/AKT kinase phosphorylated BRCA1 in vitro. We have also shown that the phosphorylation of BRCA1 by AKT occurs on the residue Thr-509, which is located in the nuclear localization signal. These results reveal a novel signaling pathway that links extracellular signals to the phosphorylation of BRCA1 in breast cancer cells.

Heregulins (NDF/neuregulin) are a group of growth factors that regulate growth, differentiation, and survival of various breast cancer cell lines (1). Heregulins activate the ErbB-2 receptor through direct binding to ErbB-3 and ErbB-4 receptors and initiate a cascade of events resulting in the stimulation of Ras/Erk and phosphatidylinositol 3-kinase (PI3K) pathways (1, 2). PI3K appears to regulate the phosphorylation and consequently the activity of the p70S6 kinase, protein kinase C isoforms, and the serine/threonine kinase AKT (3, 4). AKT activity is regulated both by binding of PI3K lipid products to its pleckstrin homology (PH) domain and by phosphorylation of Thr-308 and Ser-473 residues located within its activation loop and the C terminus, respectively (4). Activated AKT provides a survival signal that protects cells from apoptosis and mediates growth factor-induced cell proliferation (3–5). The aberrant expression of AKT has also been implicated in cell transformation (6). However, the regulation of AKT activity by heregulin and its potential importance in breast cancer cells are not known.

The hereditary breast cancer susceptibility gene product BRCA1 (7–10) has been shown to have tumor suppressive activity (11, 12) and to play a role in the differentiation of mammary epithelial cells (13, 14), apoptosis (15), and DNA recombination (16, 17). Despite several lines of evidence suggesting that serine phosphorylation of BRCA1 during cell cycle progression and in response to DNA-damaging agents may affect its function (16–19), the signaling pathway(s) involved in BRCA1 phosphorylation is unclear.

Here, we have studied the regulation of AKT activity by heregulin and its impact on the phosphorylation of BRCA1 in T47D cells. We show that heregulin stimulates phosphorylation of BRCA1 via PI3K/AKT in breast cancer cells.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Cell Cycle Analysis—T47D cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.), insulin (5 μg/ml), and antibiotics. Prior to heregulin stimulation, cells were starved for 24 h in serum-free medium. 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (Life Technologies, Inc.). T47D cells were then transiently transfected with LipofectAMINE following the protocol provided by the supplier (Life Technologies, Inc.), and 293T cells were transfected by using the CaCl2-phosphate method. Flow cytometry (>10,000 cells/sample) was used to evaluate the cell cycle profile.

Plasmids—The K227E p110 (28) and HA-BRCA1 (16) plasmids have been described elsewhere. The full-length AKT cDNA was amplified from the total RNA of HeLa cells by using reverse transcriptase-PCR and then subcloned into the Bluescript plasmid. To create the pLNCX-HA-AKT construct, AKT cDNA lacking an ATG start codon was re-amplified from the Bluescript plasmid by using a T3 primer and an AKT primer containing a Kozak and the HA sequence as well as a BamHI site. The amplified fragment was inserted into the ClaI and BglII sites of the modified pLNCX plasmid. The kinase inactive mutant HA-AKT-K179M was generated by PCR-mediated site-directed mutagenesis. The mutagenesis converted lysine 179 to a methionine and was confirmed by DNA sequencing. The AKT substrate GLAS was created by cloning of the double-stranded primers containing the AKT consensus site GIL into the expression vector pEGX-5X3 (Amersham Pharmacia Biotech). To prepare GST-BRCA1 fusion proteins, BRCA1 fragments, generated by PCR, were cloned into the modified pLNCX plasmid. The kinase inactive mutant HA-AKT-K179M was generated by PCR-mediated site-directed mutagenesis. The mutagenesis converted lysine 179 to a methionine and was confirmed by DNA sequencing. The AKT substrate GLAS was created by cloning of the double-stranded primers containing the AKT consensus site GIL into the expression vector pEGX-5X3 (Amersham Pharmacia Biotech). To prepare GST-BRCA1 fusion proteins, BRCA1 fragments, generated by PCR, were cloned into the
vector pGEX-T2 (Amersham Pharmacia Biotech) between sites BamHI and EcoRI. GST-BRCA1-3M was constructed by changing threonine at position 509 to alanine by PCR-mediated site-directed mutation. The PCR-derived constructs were confirmed as correct by direct sequencing.

Competent Escherichia coli JM109 was transformed, and recombinant clones were screened by SDS-PAGE analysis of overexpressed fusion proteins and by restriction enzyme analysis. GST fusion proteins were produced by 10 ms iso-propyl β-thiogalactopyranoside induction and purified on a large scale by affinity chromatography on glutathione-Sepharose beads. The proteins were eluted with 10 ms glutathione followed by concentration in a Centricon 30 filter (Amicon), and the buffer was exchanged to 5 mM NaPO₄ and 100 mM KCl, pH 7.4.

To produce the GST-AKT and the GST-AKT-K179M (kinase-dead) proteins in baculovirus cells, 1.8 × 10⁶ SF-9 cells were plated on a 175-cm² flask and allowed to attach for 1 h. The media were removed, and 4 ml of high titer GST-AKT baculovirus was incubated with the cells for 1 h at 27°C. Next, 20 ml of medium (SF900II supplemented with 10% FCS) was added to the cells (virus was not removed). 48 h post-infection, cells were scraped off the flask, spun down, washed with phosphate-buffered saline, and lysed in Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol). Lysate was spun at 14,000 × g, and the supernatant was mixed with glutathione beads. Beads were washed four times with phosphate-buffered saline, and GST-AKT was then eluted with free glutathione. The eluted proteins were adjusted to 40% glyceral and stored at −20°C.

Metabolic Labeling, Immunoprecipitation, and Kinase Assay—Serum-starved T47D cells were either untreated or treated with heregulin (hHRG1) (10 nm) for 30 min. Metabolic labeling experiments were performed as described (29). Whole cell extracts from 293T and T47D cells were prepared in cell lysate buffer as described (17). Immunoprecipitation and Western blot analysis of BRCA1 were performed by using either monoclonal BRCA1-17F8 (Genetex, San Antonio, TX), monoclonal D-9 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or polyclonal C-20 (Santa Cruz Biotechnology Inc.) antibodies. Proteins were separated by 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Immobilon-P membranes, and immunoblotted with one of the anti-BRCA1 antibodies by utilizing the ECL detection system. Proteins in baculovirus cells, 1.8 × 10⁶ GST-AKT baculovirus was incubated with the lysates of serum-starved T47D cells (lane 1) from total cell lysates. BRCA1 immunoprecipitates were performed as described under “Experimental Procedures.”

RESULTS AND DISCUSSION

Treatment of T47D Cells by Hererugin Leads to Phosphorylation of BRCA1—Hererugin overexpression has been shown to induce aggressive tumor growth in breast cancer cells by activating the ErB-B-2, ErB-B-3, and ErB-B-4 receptor signaling cascades (20–22). Furthermore, amplification or overexpression of the ErB-B-2 oncogene is associated with a poor prognosis in breast and ovarian cancer patients (23). The mechanism by which hererugin induces cell growth in breast cancer cells is not well understood. To investigate whether the tumor suppressor protein BRCA1 is a downstream target of the ErB-B signaling pathway in breast cancer cells, BRCA1 immunoprecipitates were prepared from whole cell lysates of serum-starved T47D cells untreated or treated with heregulin for different time periods. Expression level of BRCA1 in T47D cells is similar to that in other breast cancer cell lines such as MCF-7. However, ErB-B-2 receptors are not overexpressed in T47D cells. As shown in Fig. 1A, BRCA1 was detected as an 220-kDa protein from the lysates of serum-starved T47D cells (lane 1). This protein was not seen when control rabbit IgG was used in immunoprecipitation experiments (data not shown). Within 30 min of treatment, hererugin caused a significant decrease in the mobility of BRCA1 and gradually increased the density of

FIG. 1. Hererugin stimulation leads to phosphorylation of BRCA1 by activating the PI3K pathway. A, growth-arrested T47D cells were untreated or treated with heregulin (10 nm). Cells were harvested at the indicated time points and BRCA1 was immunoprecipitated (IP) from total cell lysates. BRCA1 immunoprecipitates were performed as described (29). Whole cell extracts from 293T and T47D cells were prepared in cell lysate buffer as described (17). Immunoprecipitation and Western blot analysis of BRCA1 were performed by using either monoclonal BRCA1-17F8 (Genetex, San Antonio, TX), monoclonal D-9 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or polyclonal C-20 (Santa Cruz Biotechnology Inc.) antibodies. Proteins were separated by 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Immobilon-P membranes, and immunoblotted with one of the anti-BRCA1 antibodies by utilizing the ECL detection system. Anti-BRCA1 immunoprecipitates were washed in extraction buffer without phosphatase inhibitor and treated with X-phosphatase (New England BioLabs, Beverly, MA) as described (19).

The total amounts of AKT and Ser-473 phosphorylation of AKT were detected by Western blot analysis. For Ser-473 phosphorylation of AKT, a phospho-specific AKT polyclonal antibody (S2437AKT), which recognizes AKT only when phosphorylated at Ser-473 (New England BioLabs), was used. The level of AKT expression was monitored by using a polyclonal antibody that recognizes AKT independently from its phosphorylation state (New England BioLabs). The level of AKT expression was monitored by using a polyclonal antibody that recognizes AKT independently from its phosphorylation state (New England BioLabs).

AKT was immunoprecipitated from total cell extracts with the C-20 antibody, and the kinase activity was assayed as described (24), except GLAS was used as a substrate.

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The upper band as compared with the untreated cells (compare lane 1 with lanes 2 and 3). It appears that the decreased migration of BRCA1 in hererugin-treated cells is due to phosphorylation, because treatment with phosphatase converted the slower migrating form to a faster migrating, single protein band on SDS-PAGE (lane 4). To confirm that the 220-kDa protein that is phosphorylated in response to hererugin treatment is BRCA1, T47D cells were transiently transfected with HA-BRCA1. Because an anti-HA antibody was used for Western blot analysis, an approximately 220-kDa protein was detected in cells transfected with the HA-BRCA1 (Fig. 1B, lane 1), whereas no such band was found in vector-transfected cells (data not shown). Consistent with the data obtained above, heregulin treatment caused a decrease in the mobility of HA-BRCA1 (lane 1 compared with lane 2). Thus, we concluded that the 220-kDa protein observed in the anti-BRCA1 immunoprecipitates is BRCA1.

Phosphorylation of BRCA1 in response to hererugin most likely occurs on serine/threonine residues, because an antiphosphoryosine antibody failed to recognize BRCA1 after hererugin treatment, whereas the same antibody detected tyrosine phosphorylation of ErB-B-2 and ErB-B-3 receptors in the same extracts (data not shown). To directly determine whether BRCA1 was phosphorylated in heregulin-treated T47D cells, we assayed in vivo 32P incorporation into BRCA1 immunoprecipitated from [32P]orthophosphate-labeled cells (Fig. 1C). The amount of 32P incorporated into the BRCA1 protein increased in response to hererugin within 30 min (Fig. 1C, lane 2 compared with lane 3), whereas no significant change was observed in the expression level of BRCA1 (data not shown).

Phosphorylation of BRCA1 Is Cell Cycle-independent—BRCA1 phosphorylation has been reported to increase during the S phase of the cell cycle (16–19). To check whether hererugin...
lin-induced phosphorylation of BRCA1 correlates with S phase entry, we analyzed the DNA content of serum-starved T47D cells over 24 h following heregulin stimulation. As shown in Table I, treatment of cells with heregulin for 30 min did not lead to any change in the percentage of the cell population arrested in the G0/G1 phase of the cell cycle, although significant BRCA1 phosphorylation had occurred. A gradual exit from the G0/G1 phase was observed beginning 6 h after stimulation with heregulin. The phosphorylation of the retinoblastoma protein, RB, in the same extracts was used as an additional indicator of cell cycle progression. A change in the phosphorylation of RB was observed beginning 12 h after heregulin treatment of cells (data not shown). These results show that the phosphorylation of BRCA1 by short term heregulin treatment precedes S phase entry.

**Herregulin-induced Phosphorylation of BRCA1 Is Mediated by PI3K**—We next tested which signaling pathway(s) is essential for phosphorylation of the BRCA1 protein by heregulin in T47D cells. Fig. 1D shows that in cells stimulated with heregulin for 30 min, BRCA1 underwent a mobility shift, which is consistent with the result observed in Fig. 1, A and B. The shift in mobility was partially blocked by pretreatment of cells with the PI3K inhibitor LY294002 (10 μM) (lane 4) but not with the MEK inhibitor PD98059 (20 μM) (lane 3). As tested under the same conditions, heregulin-induced MAP kinase activity was inhibited by PD98059 (data not shown), suggesting that the PI3K pathway, but not the MAP kinase pathway, is required for the heregulin-induced phosphorylation of BRCA1. However, because the shift in mobility of BRCA1 was blocked partially by the PI3K inhibitor LY294002, this suggests that a component of BRCA1 phosphorylation might be PI3K independent.

The hypothesis that heregulin induces BRCA1 phosphorylation in T47D cells by enhancing the activity of PI3K was further tested by transient expression of a constitutively active p110 subunit (K227E) of PI3K in 293T cells. The mobility of BRCA1 immunoprecipitated from these cells was compared with cells transfected with the vector alone. As shown in Fig. 2, transfection with the Myc-tagged K227E p110 plasmid increased the migration of BRCA1 on SDS-PAGE (Fig. 2A) and increased the activity of the endogenous AKT as detected by an increase in the SER473 phosphorylation (Fig. 2B). Expression levels of K227E p110 (Fig. 2C) and the endogenous AKT (Fig. 2D) were determined by Western immunoblotting using anti-Myc and anti-AKT antibodies, respectively. These results demonstrate that signaling initiated by activated PI3K is sufficient for phosphorylation of BRCA1 in the absence of heregulin.

**Herregulin Treatment Induces Activation of AKT in Breast Cancer Cells**—The results presented above strongly suggest that AKT, the downstream target of PI3K, might mediate BRCA1 phosphorylation in response to heregulin. To better understand the regulation of the PI3K/AKT signaling pathway by activation of ErbB receptors in breast cancer cells, growth-arrested T47D cells were treated with heregulin. Phosphorylation as well as activation of AKT were analyzed by using a phospho-specific antibody (which recognizes AKT only when phosphorylated at the Ser-473 residue) and by *in vitro* kinase assays, respectively. As shown in Fig. 3A, neither phosphorylation (upper panel) nor kinase activity (middle panel) of AKT was observed in unstimulated serum-starved T47D cells. However, stimulation with heregulin dramatically increased both the phosphorylation and the kinase activity of AKT within 1 min, which gradually increased during 30 min of treatment. No difference was observed in the protein levels of AKT between unstimulated and heregulin-stimulated cells as shown by an antibody recognizing AKT independently from its phosphorylation state (Fig. 3A, lower panel). A similar pattern of phosphorylation of AKT at Thr-308 was also observed when a phospho-specific antibody that recognizes AKT only when phosphorylated at the Thr-308 was used (data not shown).

Although previous studies suggested that AKT activation by various growth factors is PI3K-dependent, other mechanisms leading to AKT activation have also been reported (3, 4). To explore the mechanism of AKT activation by heregulin in T47D cells, the PI3K inhibitors wortmannin, LY294002, and the MAP kinase kinase (MEK) inhibitor PD98059 were used. As shown in Fig. 3B, both heregulin-induced phosphorylation and activation of AKT were completely blocked by pretreatment of cells with LY294002 (10 μM) but not with PD98059 (20 μM), which blocks MAP kinase activation by heregulin. These results demonstrate that PI3K activation is required for the phosphorylation on residue Ser-473 and for the activation of AKT.

**Herregulin-induced Phosphorylation of BRCA1 Is Blocked by the Kinase-inactive AKT**—To analyze the importance of AKT in heregulin-induced phosphorylation of BRCA1 in *vivo*, T47D cells were co-transfected with HA-BRCA1 and the kinase-inactive form of AKT, HA-AKT-K179M, carrying a point mutation that renders the kinase inactive. The AKT-K179M has been demonstrated to have a dominant-inhibitory effect toward wild-type AKT kinase activity (24–26). As shown in Fig. 4, treatment with heregulin caused a shift in the mobility of HA-BRCA1 (compare lanes 2 and 3), whereas in cells co-transfected with HA-AKT-K179M, herregulin treatment did not

| Heregulin | G0/G1 | S  | G2 + M |
|----------|------|----|--------|
|          | %    | %  | %      |
| 0 min    | 93.1 | 6.1 | 0.8    |
| 5 min    | 94.3 | 5.1 | 0.6    |
| 30 min   | 92.1 | 7.0 | 0.8    |
| 6 h      | 82.9 | 12.1| 5.0    |
| 12 h     | 79.1 | 18.4| 2.5    |
| 18 h     | 74.6 | 22.3| 3.1    |
| 24 h     | 66.8 | 31.1| 2.0    |
Heregulin Induces BRCA1 Phosphorylation

Heregulin-induced activation of PI3K leads to AKT activation and phosphorylation at Ser-473. A, serum-starved T47D cells were either untreated (0) or treated with heregulin (10 nM) for 1, 15, and 30 min. Cells were harvested, and a phospho-specific antibody that detects phosphorylated Ser-473 on AKT (anti-SER473AKT) (New England BioLabs) was employed to determine AKT phosphorylation by Western blot (WB) analysis (upper panel). AKT kinase assay was performed in immunoprecipitates prepared from untreated or treated T47D cells using GLAS as a substrate (middle panel). Expression levels of AKT were monitored by using anti-AKT antibody (New England BioLabs) (bottom panel). B, T47D cells, serum-starved for 24 h, were treated (+) or untreated (−) with heregulin (10 nM) with (+) or without (−) preincubation with LY294002 (10 μM) or PD98059 (20 μM). SER473 phosphorylation of AKT (upper panel), AKT kinase assay (middle panel), and protein levels of AKT (lower panel) were detected as described for A.

Heregulin-induced phosphorylation of BRCA1 is blocked by an inactive AKT kinase. T47D cells were transiently transfected with the HA-BRCA1 expression vector (2 μg) alone or together with the HA-AKT-K179M (4 μg) construct. Serum-starved cells were untreated (−) or treated (+) with heregulin for 30 min. Expression and mobility of HA-BRCA1 (upper panel) and HA-AKT-K179M (lower panel) were detected using F-7 anti-HA antibody (Santa Cruz Biotechnology Inc.) in a Western blot (WB) analysis.

Because a change in the mobility of HA-BRCA1 (lane 1). The expression of HA-AKT-K179M was monitored by immunoblots using an HA-specific antibody (Fig. 4, lower panel). These results reveal that activation of the PI3K/AKT pathway is necessary and sufficient to phosphorylate BRCA1 in vitro and suggest that AKT mediates BRCA1 phosphorylation in response to heregulin.

AKT Phosphorylates BRCA1 on Thr-509 Located in the Nuclear Localization Signal—To test whether AKT functions as a BRCA1 kinase in vitro, BRCA1 was immunoprecipitated from serum-starved T47D cells, and its phosphorylation by AKT was assayed in an immunocomplex kinase assay. The GST-AKT kinase, as well as the GST-AKT-K179M, were expressed in the SF-21 baculovirus system and then purified. Whereas GST-AKT significantly induced BRCA1 phosphorylation, the kinase-inactive form of AKT, GST-AKT-K179M, failed to phosphorylate BRCA1 (Fig. 5A).

To identify the phosphorylation domain of BRCA1 by AKT, we created six overlapping BRCA1 fragments spanning the entire BRCA1 open reading frame as GST fusion proteins. Approximately equal amounts of each GST-BRCA1 protein were incubated with the recombinant AKT, purified from baculovirus in vitro. As shown in Fig. 5B, lanes 3 and 8, mainly a fragment spanning amino acids 428–683, which is necessary and sufficient for interaction with AKT in vivo (data not shown), underwent phosphorylation with GST-AKT but not with kinase-inactive GST-AKT (lane 7). Interestingly, an AKT consensus sequence RXRXXTS is found in this fragment. Because the Thr-509 seems to be the most likely primary residue phosphorylated by AKT, a GST-BRCA1 fusion protein (GST-BRCA1#3M) was generated in which Thr-509 was converted to alanine. As seen in Fig. 5B, mutation of this residue greatly diminishes the phosphorylation by AKT, demonstrating that Thr-509 is the primary site of induced phosphorylation of BRCA1. However, heregulin/PI3K/AKT-dependent phosphorylation of other phosphorylation sites of BRCA1 may also be affected and may have a low level of phosphorylation upon heregulin stimulation. AKT phosphorylates BRCA1 on Thr-509 (compare lanes 12 and 13). Phosphorylation of BRCA1#3 by AKT seems to be comparable with the phosphorylation of the AKT substrate GLAS, containing the ideal AKT consensus sequence (27) fused to the GST protein.

In conclusion, our results define a pathway by which activation of ErbB receptors by heregulin can regulate the phosphorylation of BRCA1 through PI3K/AKT in breast cancer cells and indicate that AKT may be a BRCA1 kinase in vivo. The localization of the AKT phosphorylation site in the nuclear translocation signal of BRCA1 suggests that phosphorylation by AKT may interfere with the nuclear translocation and consequently with the biological activity of BRCA1. Understand-
ing the regulation of BRCA1 function by growth factors and their oncogenic membrane receptors may lead to new approaches for the treatment and prevention of both hereditary and sporadic breast cancers.

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