Breast Cancer–Associated *PIK3CA* Mutations Are Oncogenic in Mammary Epithelial Cells

Steven J. Isakoff,[1,3] Jeffrey A. Engelman,[2,4,5] Hanna Y. Irie,[1,3] Ji Luo,[2,5] Saskia M. Brachmann,[2,5] Rachel V. Pearline,[1] Lewis C. Cantley,[2,5] and Joan S. Brugge[1]

Departments of [1]Cell Biology and [2]Systems Biology, Harvard Medical School; [3]Department of Medical Oncology, Dana-Farber Cancer Institute; [4]Massachusetts General Hospital Cancer Center; and [5]Division of Signal Transduction, Beth Israel Deaconess Medical Center, Boston, Massachusetts

Abstract

Activation of the phosphoinositide 3-kinase (PI3K) pathway has been implicated in the pathogenesis of a variety of cancers. Recently, mutations in the gene encoding the p110α catalytic subunit of PI3K (*PIK3CA*) have been identified in several human cancers. The mutations primarily result in single amino acid substitutions, with >85% of the mutations in either exon 9 or 20. Multiple studies have shown that these mutations are observed in 18% to 40% of breast cancers. However, the phenotypic effects of these *PIK3CA* mutations have not been examined in breast epithelial cells. Herein, we examine the activity of the two most common variants, E545K and H1047R, in the MCF-10A immortalized breast epithelial cell line. Both variants display higher PI3K activity than wild-type p110α yet remain sensitive to pharmacologic PI3K inhibition. In addition, expression of p110α mutants in mammary epithelial cells induces multiple phenotypic alterations characteristic of breast tumor cells, including anchorage-independent proliferation in soft agar, growth factor–independent proliferation, and protection from anoikis. Expression of these mutant p110α isoforms also confers increased resistance to paclitaxel and induces abnormal mammary acinar morphogenesis in three-dimensional basement membrane cultures. Together, these data support the notion that the cancer-associated mutations in *PIK3CA* may significantly contribute to breast cancer pathogenesis and represent attractive targets for therapeutic inhibition. (Cancer Res 2005; 65(23): 10992-11000)

Introduction

The phosphoinositide 3-kinase (PI3K) family of lipid kinases is implicated in oncogenesis in many different cellular paradigms (1). Type 1a PI3Ks are composed of a heterodimer with a p85 regulatory subunit and a p110 catalytic subunit (2). Stimulation of cells by a variety of extracellular signals activates PI3K by recruitment to specific phosphotyrosine residues on signaling molecules via the two SH2 domains of the p85 subunit. This interaction results in increased catalytic activity and localizes PI3K to the site of its membrane-bound phosphatidylinositol substrates to generate phosphatidylinositol-3,4-bisphosphate [PI(3,4)P_2] and phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P_3]. These lipid second messengers then activate downstream pathways involved in many fundamental cellular processes, including proliferation, cell survival, motility, and cell growth (2). The PI3K pathway is negatively regulated by the PTEN phosphatase, which removes the 3-phosphate from PI(3,4)P_2 and PI(3,4,5)P_3, turning off the signaling cascade (3).

Dysregulation of the PI3K pathway is implicated in many cancers. Loss-of-function mutations in *PTEN* result in hyperactivity of the PI3K pathway. Such mutations are found in a variety of cancers, including breast, lung, prostate, endometrial, melanoma, glioblastoma, and lymphoma (4). Germ line mutations in *PTEN* are found in several genetic syndromes, such as Cowden disease, which predisposes patients to breast, thyroid, and other cancers (3). The serine/threonine kinase Akt/protein kinase B (PKB), a downstream target of PI3K activation, was initially identified as a retroviral oncogene that leads to its constitutive activation (5) and is amplified or up-regulated in gastric (6), pancreatic (7), and ovarian and breast (8, 9) cancers. Recently, oncogenic somatic mutations in the gene encoding the p85 regulatory subunit have been identified in colon and ovarian cancers in which a short deletion leads to constitutive PI3K activation (10). Amplification of the gene encoding the p110α subunit of PI3K (*PIK3CA*) occurs in ovarian (11), cervical (12, 13), head and neck (14), non–small cell lung (15), and esophageal (16) cancers. However, no such amplification has been identified in breast cancer (11).

Recently, somatic mutations in *PIK3CA* were identified in colon, brain, gastric, breast, and lung cancers (17). Several subsequent studies reported that 18% to 40% of breast cancer samples harbor mutations in *PIK3CA* (18–22). Although >25 mutations in *PIK3CA* were identified, the majority lie in two hotspot regions, including the central helical domain encoded by exon 9 and the COOH-terminal kinase domain encoded by exon 20. The two most common mutations led to amino acid changes of E545K in the helical domain and H1047R in the kinase domain. This raised the intriguing possibility that such mutations may result in increased PI3K activity *in vivo*. Indeed, overexpression of the H1047R mutant in NIH3T3 cells resulted in increased *in vitro* PI3K activity (17). Additionally, overexpression of these PI3K mutants in chicken embryo fibroblasts and NIH3T3 cells resulted in increased PI3K activity and transformation (23, 24).

The high frequency of *PIK3CA* mutations reported in breast cancer suggests that they may play an important role in breast cancer pathogenesis. We therefore sought to examine the phenotypic alterations induced by stable expression of these cancer-associated PI3K mutants in nontransformed mammary epithelial cells. We report here that mammary epithelial cells harboring either of the two most common *PIK3CA* mutations, E545K and H1047R, display constitutive activation of Akt and phenotypes that model certain hallmarks of cancer, including anchorage-independent growth in soft agar, protection from

Note: S.J. Isakoff and J.A. Engelman contributed equally to this work.

Requests for reprints: Joan S. Brugge, Department of Cell Biology, Harvard Medical School, Building C, Room 513, 240 Longwood Avenue, Boston, MA 02115. Phone: 617-432-3973; Fax: 617-432-3969; E-mail: joan_brugge@hms.harvard.edu.

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anoikis, and growth factor–independent growth. In addition, expression of these mutations induces abnormal acinar formation using a three-dimensional mammary morphogenesis model and results in increased resistance to chemotherapy.

Materials and Methods

Cell lines and cell culture. MCF-10A cells were obtained from American Type Culture Collection (Manassas, VA) and cultured as described (25) in growth medium containing DMEM/F-12 (Invitrogen, Carlsbad, CA) supplemented with 5% donor horse serum (HS), 20 ng/mL epidermal growth factor (EGF), 10 μg/mL insulin, 100 μg/mL hydrocortisone, 1 ng/mL cholera toxin, 50 units/mL penicillin, and 50 mg/mL streptomycin. Where indicated, cells were cultured in starvation medium (growth medium lacking EGF and insulin). MCF-10A cells expressing the p110α variants were generated by retroviral gene transfer. Hemagglutinin (HA)–tagged bovine PIK3CA cDNA was subcloned into the pDNR-Dual vector (Clontech, Mountain View, CA), and site-directed mutagenesis (Quick Change, Stratagene, La Jolla, CA) was used to generate the E545K and H1047R variants (primers available upon request). All variant cDNAs were shuttled into the JP1520 retroviral vector (26)[6] using the BD Creator System (Clontech). Vesicular stomatitis virus–pseudotyped retroviruses were generated as described previously (27) and used to infect MCF-10A cells. Stable populations were obtained by selection with 2 μg/mL puromycin (Sigma-Aldrich, St. Louis, MO).

Immunoblots. Cell lysates were prepared in NP40 lysis buffer [50 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, 1% NP40, 10% glycerol] supplemented with leupeptin (2 μg/mL), aprotinin (2 μg/mL), phenylmethylsulfonyl fluoride (PMSF; 1 mmol/L), NaF (50 mmol/L), and Na_3VO_4 (1 mmol/L). Lysates were cleared by centrifugation at 16,000 × *g* for 15 minutes at 4°C and the supernatant was used for all subsequent procedures. Lysates were separated by SDS-PAGE and transferred to either nitrocellulose or polyvinylidene difluoride membranes. Immunoblotting was done according to the antibody manufacturers' recommendations using enhanced chemiluminescence. Antibodies used for immunoblotting were HA.11 (Covance, Berkeley, CA); anti-p-Akt (S473), anti-p70S6K (T389), anti-S6, anti-eIF4e, anti-p110α, and anti-p-Erk (Cell Signaling, Beverly, MA); and anti-Erk, anti-Akt1, and anti-p85 (Upstate Biotechnology, Lake Placid, NY). In preliminary experiments, we noted that in MCF-10A cells the addition of insulin to the starvation medium had no effect on p-Akt or p-Erk levels. For simplicity, unless specifically noted in the text, we used starvation medium lacking both EGF and insulin. Cells were cultured in starvation medium for 24 hours or for the indicated time before lysis and stimulated for 10 minutes with full growth medium containing EGF and insulin as indicated. LY294002 and rapamycin (Calbiochem, San Diego, CA) in DMSO were added to cells 1 hour before lysis as indicated.

Phosphoinositide 3-kinase assay. The assay was done as described previously with minor modifications (28). Briefly, MCF-10A cells were lysed with lysis buffer [25 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40, 1 mmol/L EDTA, 20 mmol/L NaF, 10 mmol/L $NaPP_i$, 50 mmol/L DTT, 1 mmol/L Na_3VO_4, 1 mmol/L PMSF, 4 μg/mL each of leupeptin, aprotinin, and pepstatin]. The lysate was clarified by centrifugation, and PI3K was immunoprecipitated overnight at 4°C with anti-HA antibody. The immunoprecipitate was captured on protein G-Sepharose beads and washed thrice with immunoprecipitation wash buffer [25 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40, 1 mmol/L EDTA, 20 mmol/L NaF, 10 mmol/L $NaPP_i$, 50 mmol/L β-glycerophosphate] and thrice with kinase assay buffer [20 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, 0.5 mmol/L EGTA]. The immunoprecipitated PI3K was preincubated with a lipid substrate mix of phosphatidylserine and phosphatidylinositol [in 30 mmol/L HEPES (pH 7.4), 1 mmol/L EGTA] at room temperature for 10 minutes. Kinase reaction was initiated with the addition of [γ-^{32}P]ATP [in 30 mmol/L HEPES (pH 7.4), 10 mmol/L $MgCl_2$, 0.5 mmol/L ATP] and allowed to proceed for 10 minutes at room temperature before termination with 4 mol/L HCl. The phospholipid products were extracted with methanol/$CHCl_3$ (1:1). The organic fraction contained the phospholipids that were separated by TLC with 2 mol/L acetic acid/*n*-propyl alcohol (35:65) as solvent. Phospholipid signal was quantified using a PhosphoImager screen. For experiments assessing inhibition by LY294002 *in vitro*, the inhibitor was added to the lipid mix to yield the final indicated concentrations.

Growth curves and fluorescence-activated cell sorting. MCF-10A cells were seeded in triplicate at 25 × 10^3 per well in 12-well plates on day 0 in growth medium lacking EGF. Cells were trypsinized and counted with a hemacytometer on days 1, 4, 7, and 10. Fresh medium was changed on the same days. For cell cycle analysis by fluorescence-activated cell sorting (FACS), cells were seeded at 500,000 per 10-cm plate in growth medium for 24 hours and then changed to either fresh growth medium or growth medium lacking EGF for another 24 hours. Cells were harvested by incubating the cells at 37°C in PBS with 2.5 mmol/L EDTA for 30 minutes. Following EDTA treatment, the cells were fixed in 80% ethanol for 15 minutes at 4°C. After fixation, the cells were washed with PBS containing 1% goat serum and stained at 37°C for 1 hour in propidium iodide/RNase A solution (PBS with 2.5 mmol/L EDTA, 10 μg/mL propidium iodide, 0.25 mg/mL RNase A). Flow cytometry was done to determine DNA content using a BD Biosciences FACSCalibur, and data were analyzed using CellQuest (BD Biosciences, San Jose, CA).

Colony formation in soft agar assay. To assess anchorage-independent growth, triplicate samples of 5 × 10^4 cells from each MCF-10A-derived cell line were mixed 4:1 (v/v) with 2.0% agarose in MCF-10A growth medium for a final concentration of 0.4% agarose. The cell mixture was plated on top of a solidified layer of 0.5% agarose in growth medium. Cells were fed every 6 to 7 days with growth medium containing 0.4% agarose. Cells were stained with 0.02% iodonitrotetrazolium chloride (Sigma-Aldrich) and photographed after 21 days. Colonies in the entire well were counted using a dissecting microscope and colonies larger than 50 μm were included.

Anoikis assay. Detachment-induced cell death was assayed essentially as described (29). Six-well tissue culture plates were coated with 6 mg/mL poly-HEMA in 95% ethanol, incubated at 37°C for several days until dry, and rinsed with PBS. MCF-10A cells were then plated at 400,000 per well in 2 mL growth medium for 42 hours. Cells were collected and washed with PBS. Apoptosis was measured using a Cell Death Detection ELISA kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Cells were lysed in 600 μL lysis buffer supplied with the kit and 20-μL lysate was used in the assay. Absorbance at 405 nm was measured and is reported with arbitrary units. In parallel, 2 × 10^6 cells were plated in poly-HEMA-coated 10-cm plates for 42 hours, lysed, and analyzed by Western blot as described above.

Morphogenesis assay. The three-dimensional culture of MCF-10A cells on basement membrane was carried out as described previously (25, 30). Briefly, 5 × 10^3 cells were resuspended in modified growth medium containing 2% Matrigel (BD Biosciences), 2% HS, and 5 ng/mL EGF plus all other additives and seeded on top of a layer of growth factor–reduced Matrigel. Medium was exchanged every 4 days. Rapamycin (20 nmol/L) was added to cultures as indicated on day 2 of three-dimensional culture and exchanged every 4 days thereafter. Photographs of representative fields were taken on day 6.

Cell viability assay. MCF-10A cells were seeded in 96-well plates at 10^4 per well in 100 μL growth medium and allowed to adhere overnight. The next day, paclitaxel was added by 2-fold serial dilutions. After 24 hours, cell viability was quantified by MTS assay using the CellTiter 96 Aqueous kit (Promega, Madison, WI) according to the manufacturer's instructions. Untreated wells of each cell line were used as the control for calculation of percent viability. Wells were plated in at least triplicate for each experiment.

Results

Cancer-associated mutations in *PIK3CA* promote constitutive activation of Akt in mammary epithelial cells. To determine whether cancer-associated PIK3CA mutations promote growth factor–independent activation of the PI3K pathway in human mammary epithelial cells, we generated vectors encoding HA epitope–tagged p110α variants containing each of the two most

[6] J. Pearlberg, unpublished data.

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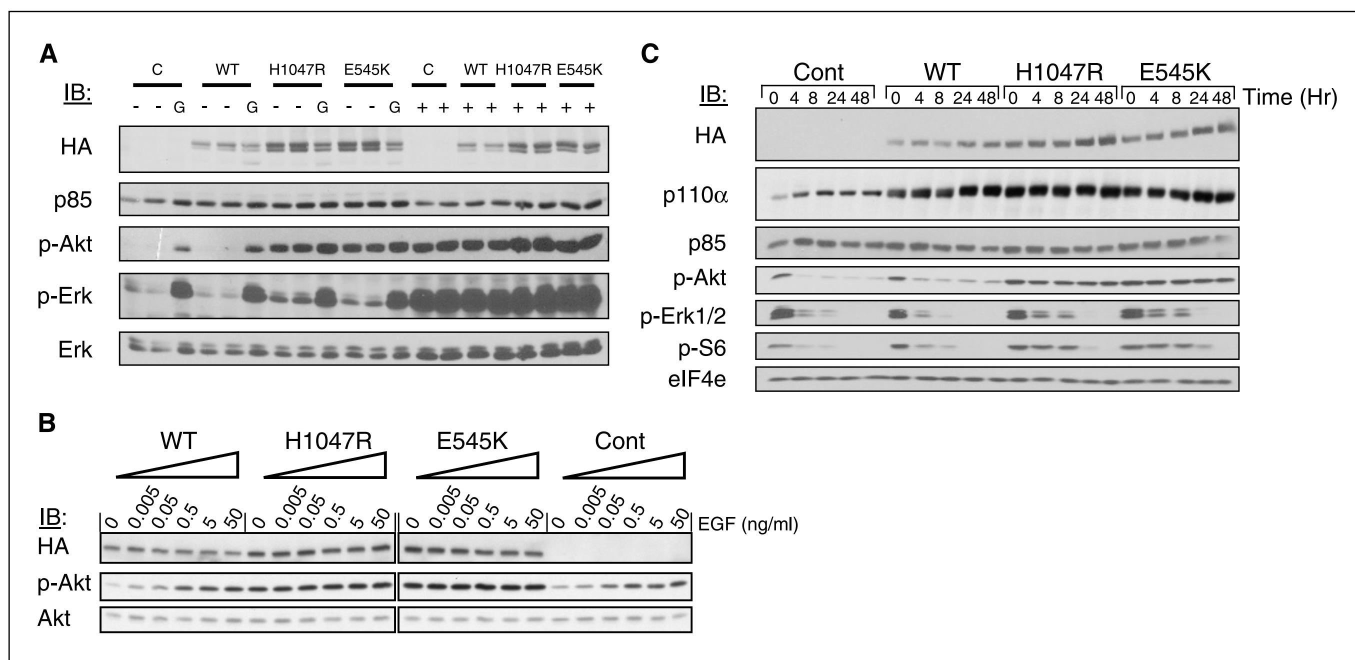

Figure 1. Constitutive activation of the PI3K pathway in mammary epithelial cells expressing p110α mutants. *A,* MCF-10A cells expressing WT or mutant (H1047R and E545K) HA-tagged p110α or vector control (*C*) were seeded in growth medium for 24 hours and then cultured in starvation medium (-) or growth medium (*G*), which contains 20 ng/mL EGF and 10 μg/mL insulin, for an additional 24 hours, lysed, and immunoblotted with the indicated antibodies. As a control, after 24 hours in starvation medium, cells were stimulated for 10 minutes with growth medium (+). *B,* cell lines as above were deprived of EGF and insulin for 24 hours and then stimulated for 10 minutes with increasing concentrations of EGF as indicated. Lysates were immunoblotted as indicated. *C,* cell lines were seeded in six-well plates in growth medium overnight, and growth medium was replaced with starvation medium lacking EGF and insulin for the indicated time points. Lysates were immunoblotted with antibodies as indicated.

common amino acid substitutions found in breast cancer, E545K and H1047R. Stable pools of MCF-10A mammary epithelial cells expressing either variant were generated by retroviral infection, and the activation of Akt/PKB was monitored by analyzing phosphorylation at S473. After 24 hours of growth factor (EGF and insulin) withdrawal, there was a significant loss of S473 phosphorylation (p-Akt) in MCF-10A cells expressing wild-type (WT) p110α and control MCF-10A cells compared with cells maintained in growth medium (Fig. 1*A*). In contrast, cells expressing mutant p110α maintained p-Akt in the absence of growth factors. When maintained in growth medium, the level of basal p-Akt was greater in the mutant p110α cell lines than in WT p110α or control cells (Fig. 1*A*). On acute growth factor stimulation, all cell lines displayed increased p-Akt. There was no difference in expression levels of p85 between control and mutant cell lines. Cells harboring PIK3CA mutations also displayed slightly increased p-Erk levels after growth factor withdrawal (Fig. 1*A*). However, the p-Erk levels promoted by the PIK3CA mutations in the absence of growth factors were lower than those observed when cells were cultured in normal growth medium.

To further evaluate the sensitivity of the mutant p110α isoforms to growth factor stimulation, a dose-response curve was generated using increasing concentrations of EGF (Fig. 1*B*). In p110α mutant-expressing cells, the basal level of p-Akt was near maximal, whereas in control and WT-expressing cells, 0.05 to 0.5 ng/mL EGF were necessary to achieve a similar relative activation (Fig. 1*B*; data not shown). In addition, the basal p-Akt in the mutant-expressing cells was greater than the maximal p-Akt in controls. Together, these results show that p110α harboring the mutations found in breast cancer functionally activate the PI3K/Akt pathway in mammary epithelial cells in an EGF- and insulin-independent manner.

To investigate the kinetics of the Akt regulation following growth factor withdrawal, we analyzed lysates collected over a time course of EGF and insulin withdrawal from the various cell lines (Fig. 1*C*). Four hours after growth factor withdrawal, control and WT cells display maximally reduced levels of p-Akt, whereas the E545K and H1047R cells have persistent p-Akt. Cells expressing the mutant p110α continue to sustain abundant levels of p-Akt for at least 48 hours after growth factor withdrawal. In addition, on growth factor withdrawal, the time course of dephosphorylation of the S6 ribosomal protein target of p70S6 kinase was slightly delayed (Fig. 1*C*). A similar delay in dephosphorylation of Erk was observed (Fig. 1*C*), in agreement with the slight enhancement of p-Erk levels observed in Fig. 1*A*. The evidence that sustained activation of Akt does not lead to maintenance of p70S6K activation suggests that Akt-independent modes of regulation of the mammalian target of rapamycin (mTOR) pathway are dominant in these conditions.

After 24 to 48 hours of EGF withdrawal, the relative expression level of the recombinant p110α increased, with the mutants increasing to a greater extent than the WT (Fig. 1*C*). This is consistent with the expression level observed in Fig. 1*A* after 24 hours of growth factor withdrawal (compare - and *G* in Fig. 1*A*, *top*). Immunoblotting with anti-p110α antibodies shows that the expression of total p110α was equivalent in the WT and mutant cell lines when cultured in normal growth medium. The p110α antibody is a monoclonal antibody raised against amino acids 100 to 300 of human p110α, which is identical to bovine p110α, except for three amino acids, and seems to recognize the endogenous human and the recombinant bovine proteins approximately equally.[7]

[7] J. Engelman, unpublished data.

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Thus, it seems that although there are equivalent levels of total p110α and p85 in the WT and mutant cell lines, on growth factor withdrawal, the mutant p110α comprises more of the total p110α. The PIK3CA cDNA is under control of the cytomegalovirus promoter in the retroviral vector, which may account for the relative increase in expression of the recombinant p110α compared with the endogenous p110α. The data above temporally dissociate the apparent induction of mutant p110α at 24 to 48 hours of growth factor withdrawal from the increased level of p-Akt present at 4 hours of withdrawal. Therefore, the increased activation of the PI3K pathway is unlikely to be explained by the slight relative increase observed in p110α after growth factor withdrawal.

The *PIK3CA* mutations confer increased *in vitro* phosphoinositide 3-kinase activity and are sensitive to pharmacologic phosphoinositide 3-kinase inhibition. To test whether introduction of the cancer-associated mutations into PIK3CA causes an increase in PI3K activity, lysates from the MCF-10A cells grown in the absence of growth factors for 24 hours were immunoprecipitated with anti-HA antibodies, and *in vitro* PI3K assays were done. The PI3K activity of the H1047R and E545K mutant proteins was 2.5- to 3.5-fold higher than WT (Fig. 2*A*). Notably, the amount of the p85 regulatory subunit of PI3K that coimmunoprecipitated with p110α was similar between mutant and WT cell lines. This shows that the PIK3CA mutations increase the intrinsic kinase activity of PI3K and further suggests that the difference in PI3K activity noted above is not due to quantitative differences in binding to p85. It does not exclude, however, that qualitative differences in protein interactions or conformations exist.

Several pharmacologic inhibitors of the PI3K family have been developed, including the fungal metabolite wortmannin and the structurally unrelated molecule LY294002, and additional PI3K inhibitors are in preclinical development (31). Increasing doses of LY294002 inhibited the *in vitro* PI3K activity of the mutant and WT isoforms similarly (Fig. 2*B*). Although the mutant p110α isoforms had greater baseline activity than untreated WT control, activity was inhibited to a similar degree (Fig. 2*B*; data not shown). To further examine whether the PI3K pathway was inhibited by LY294002 in cells, the MCF-10A cells were incubated in starvation medium overnight and analyzed for p-Akt after LY294002 treatment. In the absence of LY294002, the H1047R and E545K mutants displayed higher basal p-Akt compared with control and WT cells (Fig. 2*C*). After LY294002 treatment, the mutant cell lines lost detectable p-Akt, thereby showing that pharmacologic inhibition of cancer-associated mutant p110α results in decreased p-Akt. Furthermore, stimulation of cells for 10 minutes with EGF-induced p-Akt in all cell lines, and this induction was also blocked by LY294002. As expected, treatment of cells with the mTOR inhibitor rapamycin did not affect p-Akt. The mutant cell lines showed small but reproducible basal phosphorylation of p70S6K (pp70), which was inhibited by rapamycin and LY294002. Rapamycin and LY294002 did effectively inhibit phosphorylation of p70S6K on EGF stimulation. Together, these results show that the PI3K mutants have increased PI3K enzymatic activity, which is inhibited *in vitro* and *in vivo* by pharmacologic inhibitors of PI3K. Furthermore, rapamycin remains an effective inhibitor of the PI3K pathway downstream of mTOR. This may be important given that PIK3CA mutations are present in a significant percentage of breast cancer and rapamycin, and its analogues are currently in clinical trials assessing their efficacy as antitumor agents alone and in combination with other agents.

Mutant p110α expression permits epidermal growth factor–independent growth. Growth factor–independent proliferation is a common feature of transformed cells and is considered one of the hallmarks of cancer (32). MCF-10A cells are not transformed and

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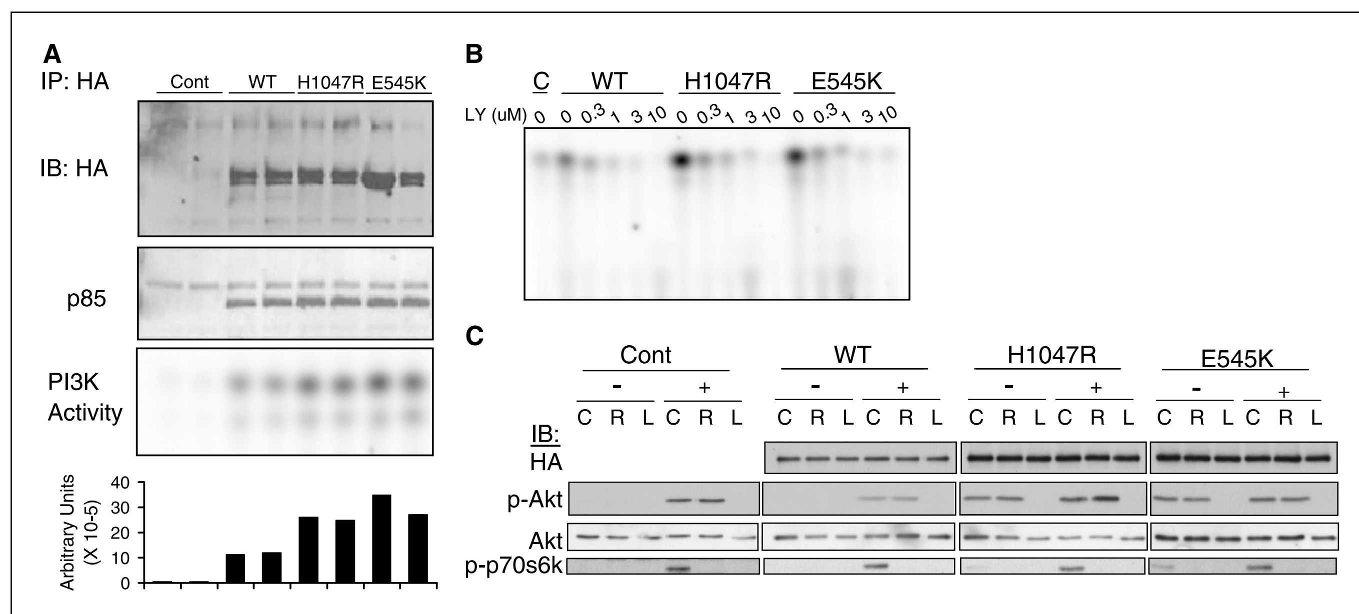

Figure 2. Increased *in vitro* PI3K activity of mutant p110α and inhibition by LY294002. *A,* MCF-10A cells as in Fig. 1*A* were lysed and p110α was immunoprecipitated with HA antibodies. Immunoprecipitates were split in half and one half was immunoblotted with anti-HA and anti-p85 as indicated (*top two*). The other half was subjected to *in vitro* PI3K assays that were quantified by PhosphoImager analysis and the data are plotted as arbitrary units (*bottom two*). Samples from each cell line were prepared and analyzed in duplicate. Representative of three independent experiments. *B,* cell lines as in (*A*) were lysed and immunoprecipitated as in (*A*). Increasing levels of the PI3K inhibitor LY294002 (*LY*) were added directly to the *in vitro* reaction at the indicated concentration, and PI3K assays were done as in (*A*). *C,* cell lines as in (*A*) were cultured in starvation medium for 24 hours. Cells were then either left in control starvation medium (*C*) or incubated for 1 hour in starvation medium containing 20 nmol/L rapamycin (*R*) or 20 μmol/L LY294002 (*L*). After 1 hour, cells were either lysed directly (-) or stimulated with growth medium (+) containing the indicated inhibitor for 10 minutes and lysed. Lysates were immunoblotted with the indicated antibodies.

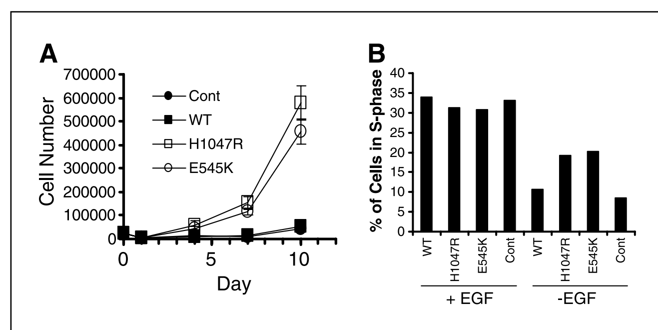

Figure 3. Effects of *PIK3CA* mutations on growth factor–independent cell growth. *A,* MCF-10A cells as in Fig. 1*A* were seeded in 12-well plates at 25×10^3 per well in growth medium lacking EGF on day 0 and cell numbers were plotted for each day indicated. Each experiment was done with triplicate wells. *Points,* mean; *bars,* SD. Representative experiment. *B,* cells were seeded at 5×10^5 per 10-cm plate overnight. Medium was then exchanged with fresh growth medium (*+EGF*) or growth medium lacking (*-EGF*) for 24 hours. Cells were then collected, fixed, and analyzed by FACS to determine the percentage of cells in the S phase of the cell cycle.

their proliferation requires EGF in the growth medium. This requirement can be overcome by exogenous expression of several oncogenes, such as activated colony-stimulating factor receptor (CSF-1R; ref. 33), activated mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (34), Ras, and Raf.[8] We tested whether expression of the activated PI3K mutants in MCF-10A cells was sufficient for EGF-independent growth. Each of the two mutant PI3K-expressing cell lines was able to proliferate in the absence of EGF, whereas the WT and control cells were not (Fig. 3*A*). In the cell lines harboring mutant PIK3CA, there was no difference in proliferation relative to WT-expressing cells when EGF was maintained in the growth medium (data not shown). To confirm this observation, the fraction of cells in the S phase of the cell cycle was determined by FACS analysis in the presence of EGF or after 24 hours of EGF withdrawal. In the absence of EGF, the PI3K mutant cells showed a 2-fold increase in the percentage of S phase cells compared with control and WT cells (Fig. 3*B*). As expected, there was no difference between mutant and WT cells grown in the presence of EGF (Fig. 3*B*). Together, these results show that expression of the cancer-associated, constitutively active PI3K mutants is sufficient to allow for EGF-independent monolayer growth of MCF-10A cells.

Cancer-associated mutations in *PIK3CA* induce anchorage-independent survival and proliferation. The ability of cells to form colonies in soft agar is a property of transformed cells that best correlates with *in vivo* tumorigenicity (35). Each of the mutant PI3K cell lines formed colonies in soft agar, but neither control MCF-10A cells nor cells expressing WT PI3K were able to do so (Fig. 4*A*). The mutants were equivalent in their ability to form colonies (Fig. 4*A*). Therefore, the cancer-associated mutations in PI3K are able to induce anchorage-independent proliferation in mammary epithelial cells.

A common feature of malignant transformation is the ability to evade apoptotic cell death signals (32). Epithelial cells and endothelial cells require extracellular matrix attachment for cell survival and undergo apoptosis when detached from the matrix through a process called anoikis (36). Malignant cells are often resistant to anoikis, and activation of the PI3K pathway has been shown to contribute to the survival of transformed cells after detachment (37). Activation of Akt has been shown to protect MCF-10A cells from anoikis (29, 38). Expression of the PI3K mutants in MCF-10A cells resulted in a 2-fold reduction in anoikis compared with control cells (Fig. 4*B*). However, expression of WT p110α failed to provide any protection. Protection from anoikis correlated with persistent p-Akt in the mutant p110α cell lines compared with control or WT (Fig. 4*B*), showing that the activated p110α mutants continued to signal through the Akt pathway in suspension.

Altered three-dimensional acinar morphogenesis induced by the *PIK3CA* mutations. Mammary epithelial cells form acinar-like structures consisting of an outer layer of polarized, growth-arrested epithelial cells surrounding a hollow lumen and basal

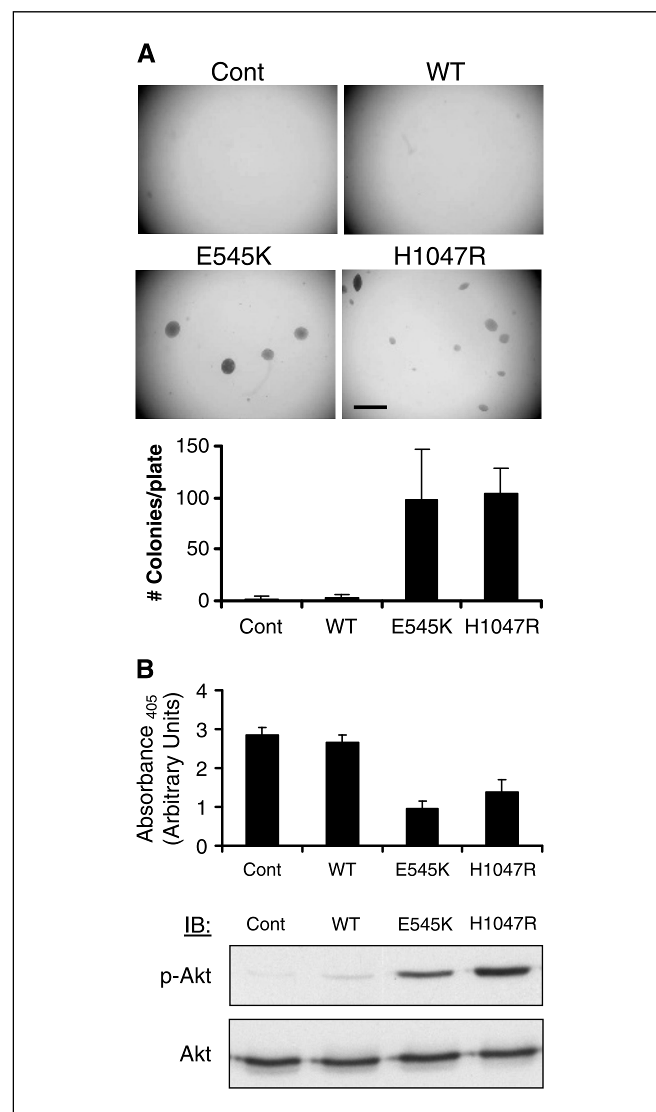

Figure 4. Effects of *PIK3CA* mutations on anchorage-independent proliferation and survival. *A,* soft agar colony formation. MCF-10A cells as in Fig. 1*A* were plated as described in Materials and Methods. *Columns,* mean of a representative experiment done in triplicate wells; *bars,* SD. Bar, 1 mm. *B,* anoikis. Cell lines were cultured in suspension in poly-HEMA-coated plates for 42 hours as described in Materials and Methods. Apoptosis was measured using a colorimetric ELISA assay to detect histone release. Results are plotted as arbitrary units of absorbance at 405 nm. Greater absorbance indicates greater apoptosis and histone release. *Columns,* mean of a representative experiment done in triplicate; *bars,* SD. Immunoblotting was done on a second set of suspended cells prepared in parallel to assess the level of p-Akt (S473).

[8] J. Brugge and A. Witt, unpublished observation.

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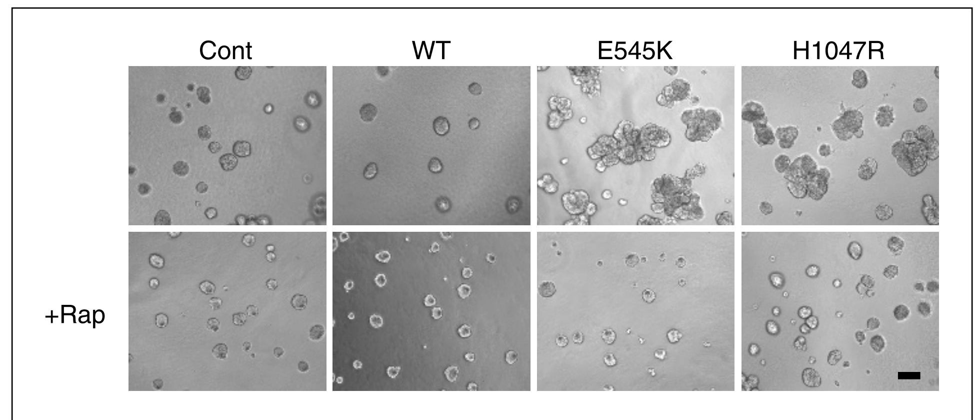

Figure 5. Effects of *PIK3CA* mutations on mammary acinar morphogenesis. MCF-10A cells as in Fig. 1*A* were cultured on a bed of Matrigel as described in Materials and Methods. Rapamycin (20 nmol/L; +*Rap*) was added (*bottom*) to culture medium on day 1 and maintained in culture medium. Representative bright-field images of acini taken on day 6. Bar, 100 μm.

deposition of basement membrane components when plated within a reconstituted basement membrane gel (25, 39). Our laboratory has shown previously the utility of this model system to study the phenotypes induced by oncogenes, such as ErbB2, CSF-1R, and Akt (30, 33, 34). Inducible activation of ErbB2 homodimers in this model results in generation of multiacinar structures with constitutively proliferating cells that fill the hollow lumens (30). Inducible activation of Akt results in large, partially filled, misshapen acini and this phenotype was effectively inhibited by the mTOR inhibitor rapamycin (34). These distorted epithelial structures are reminiscent of the aberration in glandular structures commonly observed in premalignant, noninvasive tumors.

When cultured in Matrigel, MCF-10A cells expressing the active PI3K mutants formed large, highly proliferative, abnormal structures that did not invade into the surrounding matrix (Fig. 5). In contrast, WT cells formed acini similar to control cells (Fig. 5). Treatment with rapamycin prevented the abnormal acinar morphogenesis, suggesting that the Akt/mTOR pathway is necessary for the full phenotypic effect of the activated PI3K in this model system (Fig. 5). This result is in agreement with previous data from our laboratory showing that rapamycin prevented abnormal morphogenesis induced by activated Akt (34).

***PIK3CA* mutations confer resistance to chemotherapy.** A growing body of evidence supports a role for the PI3K pathway in mediating resistance to traditional and targeted chemotherapies (40–45). We determined whether MCF-10A cells expressing the active PI3K mutants were more resistant to paclitaxel than control or WT cells. After 24 hours of exposure to varying concentrations of paclitaxel, cells expressing the active PI3K mutants had increased viability compared with control or WT cells (Fig. 6). The IC_{50} of control cells was ~8 nmol/L, whereas the IC_{50} for cells expressing activated PI3K was not reached at this time point for paclitaxel doses up to 128 nmol/L. These results support the hypothesis that somatic mutation of PIK3CA may represent another mechanism by which cancer cells develop resistance to chemotherapy and further support that inhibiting the PI3K pathway may restore chemotherapy sensitivity.

Discussion

In this study, we have evaluated the phenotypic alterations in MCF-10A mammary epithelial cells induced by expression of the recently discovered PIK3CA mutations. Our findings support the involvement of these mutations in breast tumor pathogenesis based on their ability to induce colonies in soft agar and hyperproliferative, abnormal acini in three-dimensional culture, protect from anoikis, promote growth factor–independent proliferation, and confer resistance to chemotherapy. In addition, our results indicate that these mutations promote increased PI3K activity and PI3K/Akt signaling in the absence of growth factors.

The compiled data from several studies indicate that ~25% to 30% of breast cancers harbor mutations in PIK3CA (17–22). The two most frequent mutations result in amino acid substitutions at H1047R and E545K, the two mutations used in our investigations. Interestingly, alterations leading to loss of PTEN expression are rarely observed in the same tumors as those containing PIK3CA mutations (21). This may be expected because either event results in increased 3-phosphoinositide levels. Thus, either loss of PTEN expression or PIK3CA mutation likely occurs in ~50% to 75% of breast cancers, underscoring the importance of the PI3K pathway in breast cancer and making it the most commonly mutated pathway in breast cancer known to date. The data in this article strengthen conclusions from previous studies that the cancer-associated PIK3CA mutations (17, 23, 24, 46) or membrane targeting of p110α (47) confer oncogenic activity to this key regulatory protein and this study is the first to show that expression of the two most frequently mutated variants of PIK3CA are sufficient to transform

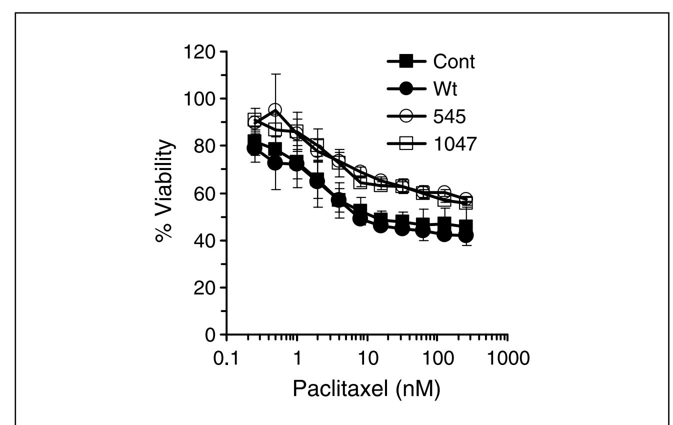

Figure 6. Effect of PIK3CA mutations on paclitaxel sensitivity. MCF-10A cells as in Fig. 1*A* were seeded at 10×10^3 per well in 96-well plates overnight. Two-fold serial dilutions of paclitaxel from 128 to 0.25 nmol/L were added at the indicated doses, and 24 hours later, cell viability was determined by MTS assay. Results are presented as the % viability of each cell line at the indicated paclitaxel dose compared with untreated cells. *Points,* mean of three independent experiments; *bars,* SD.

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mammary epithelial cells. Thus, inhibitors that specifically target PI3K may be particularly effective for the treatment of breast cancers harboring these mutations.

Expression of the activated p110α isoforms in MCF-10A cells promoted EGF-independent cell proliferation in monolayer culture. In contrast, MCF-10A cells expressing activated Akt are dependent on EGF for monolayer growth (34). This suggests that activating mutations of PI3K induce additional Akt-independent signaling pathways that are necessary for EGF-independent monolayer growth. This is consistent with previous observations that human mammary epithelial cells immortalized with hTERT and LT can proliferate in medium with reduced growth factors on expression of either myr-p110α alone or activated Akt and Rac together (47). However, activated Akt alone was not able to support growth under the same conditions.

When cultured in Matrigel, MCF-10A cells expressing mutant p110α are able to form large disorganized structures and escape the controls that normally limit their proliferation in acinar structures. In contrast to their behavior in monolayer culture, cells expressing mutant p110α were unable to proliferate in Matrigel in the absence of EGF.[9] A similar EGF dependence for three-dimensional growth was observed in cells expressing activated Akt, and the EGF requirement in those cells could be overcome by coexpression of the proliferative oncogenes human papillomavirus E7 or cyclin D1 (34). Therefore, activation of the PI3K pathway at the level of PI3K itself provides a sufficient proliferative signal to allow for monolayer growth in the absence of EGF, but the proliferative signal is not strong enough to overcome the constraints imposed in the three-dimensional morphogenesis system. Importantly, rapamycin inhibited the abnormal morphogenesis induced by activated PI3K but did not prevent the development of normal acinar structures. This suggests that the mTOR pathway is required for mediating the abnormal morphogenesis induced by activated PI3K but not normal morphogenesis. This supports the idea that inhibition of mTOR by rapamycin or its analogues may be a viable strategy in patients harboring mutations in PIK3CA.

A variety of chemotherapeutic agents converge on a common final pathway leading to apoptotic cell death. Activation of the PI3K pathway has been shown to enhance the survival of breast cancer cells in response to such agents and contribute to chemotherapy resistance (38, 40, 42, 48, 49). Here, we used a cell viability assay to show that mammary epithelial cells expressing the activated p110α isoforms found in breast cancer were more resistant to paclitaxel chemotherapy than control cells. The PI3K pathway may lead to increased resistance to paclitaxel by several mechanisms. Akt activation leads to phosphorylation of the proapoptotic protein BAD, thereby preventing its association with BCL-XL, resulting in reduced apoptosis following paclitaxel treatment in ovarian carcinoma cells (50). Furthermore, activation of PI3K has been shown to induce expression and activation of MDR-1 and MRP-1 in breast and prostate cancer cell lines (43, 51), possibly contributing to paclitaxel resistance. Recent data also suggest that regulation of glycogen synthase kinase 3β downstream of Akt may also contribute to paclitaxel sensitivity (52). Further work to elucidate the mechanisms by which the PI3K pathway may contribute to chemotherapy resistance may have important implications for clinical trials combining traditional chemotherapies with novel agents, particularly in patients harboring activated PI3K mutations. If the presence of PIK3CA mutations in breast cancer patients correlates with increased resistance to chemotherapy, then agents that target the PI3K/Akt pathway may be particularly useful to restore sensitivity in these patients. Indeed, recent preclinical data support the rationale of combining rapamycin with paclitaxel in breast cancer cell lines (53).

While this article was in preparation, Samuels et al. reported findings regarding the function of the PIK3CA mutations in colon cancer cell lines (46). In their study, they evaluated two colon cancer cell lines that harbor oncogenic PIK3CA mutations and, using gene targeting inactivation techniques, isolated clones of these cell lines that had either WT PIK3CA or mutant PIK3CA. The clones with the mutant PIK3CA had increased levels of p-Akt and p-FKHRL. In the absence of serum, the mutant clones formed more colonies in soft agar and showed greater proliferation in monolayer culture. Our investigations are indeed complementary and, together with the experiments in chicken embryo fibroblasts (23) and NIH3T3 cells (24), provide compelling evidence that mutant PIK3CA confers an oncogenic stimulus. Whereas our studies reveal that expression of these p110α mutants in nontransformed, mammary epithelial cells confers an oncogenic phenotype, their study shows that inactivation of the mutant PIK3CA oncogene renders a cancer cell line less transforming in the absence of serum.

What is the mechanism of action by which these mutations promote oncogenic phenotypes? Our studies reveal that immunoprecipitated PI3K containing the mutant p110α subunits have increased *in vitro* lipid kinase activity compared with WT PI3K (Fig. 2). These findings agree with other published reports (17, 23, 24). We also observe increased levels of p-Akt in growth factor–starved MCF-10A cells harboring PIK3CA mutations, a finding also observed by others when these mutants were expressed in fibroblasts. Thus, these mutant p110α subunits seem to result in constitutive activation of PI3K, which likely accounts for its oncogenic phenotype.

However, the molecular mechanisms by which these mutations promote increased PI3K activation remain unknown. Although one study reported that rare PIK3CA mutations in the p85-binding domain resulted in a decreased association with p85, this does not seem to be the case with the common H1047R and E545K mutations (Fig. 2). Because the total amount of PI3K holoenzyme in a cell is often limited by the available p110, it is possible that the mutations could increase PI3K signaling by promoting increased levels of p110α. However, we found no increase in the half-life of the mutant compared with WT p110α (data not shown) and the levels of total p85 and p110α were similar in MCF-10A cells expressing WT and mutant PI3K (Fig. 1*C*). Additionally, as mentioned above, the mutants have increased PI3K activity *in vitro* (Fig. 2), suggesting that the mutations lead to a structural or conformational alteration that leads to increased activity of the PI3K holoenzyme.

A recent study showed that mutation of the p85 regulatory subunit of PI3K can confer oncogenic capacity to this adaptor protein. Truncation of p85 has been observed in ovarian and colon cancers. These p85 mutations are believed to promote transformation via constitutive activation of PI3K (10). Interestingly, a recent report showed that a deletion mutant of p85 truncated at position 571 (p65), near the end of the inter-SH2 (iSH2) domain, increases PI3K activity (54). Using heteronuclear nuclear magnetic resonance spectroscopy, the authors show that the COOH-terminal end of the iSH2 domain (581-593) is closely associated with the NH_2-terminal

[9] S. Isakoff, unpublished observation.

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SH2 (NSH2) domain of p85. The NSH2 domain of p85 negatively regulates PI3K activity (55) and thus is thought to maintain PI3K in a low activity state until the NSH2 domain engages phosphotyrosine-containing proteins. The authors therefore conclude that p65 loses the packing interaction between the iSH2 domain and the NSH2 domain, thereby relieving the NSH2 inhibition of PI3K resulting in increased PI3K activity. The authors further suggest that the PIK3CA mutations, specifically E545K, may abrogate the interaction between the p85 NSH2 domain and p110α, thereby leading to increased PI3K activity. Thus, it is interesting to speculate that these PIK3CA mutations may qualitatively affect p85/p110 interactions to promote increased PI3K activity. Understanding the molecular mechanisms by which these mutations promote increased PI3K activity may facilitate the rational design of p110α inhibitors specific for the cancer-associated mutant p110α.

In summary, we have shown that expression of the recently identified mutations in PIK3CA in mammary epithelial cells results in constitutive PI3K activation and induces multiple phenotypic alterations characteristic of breast tumor cells, including anchorage-independent proliferation in soft agar, growth factor–independent proliferation, and protection from anoikis. Expression of these mutations also confers increased resistance to paclitaxel and induces abnormal mammary acinar morphogenesis in three-dimensional basement membrane cultures. Together, these data support the notion that the cancer-associated mutations in PIK3CA may significantly contribute to breast cancer pathogenesis and represent attractive targets for therapeutic inhibition.

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