Involvement of Ras Activation in Human Breast Cancer Cell Signaling, Invasion, and Anoikis

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ABSTRACT

Although mutated forms of ras are not associated with the majority of breast cancers (<5%), there is considerable experimental evidence that hyperactive Ras can promote breast cancer growth and development. Therefore, we determined whether Ras and Ras-responsive signaling pathways were activated persistently in nine widely studied human breast cancer cell lines. Although only two of the lines harbor mutationally activated ras, we found that five of nine breast cancer cell lines showed elevated active Ras-GTP levels that may be due, in part, to HER2 activation. Unexpectedly, activation of two key Ras effector pathways, the extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase and phosphatidylinositol 3-kinase/AKT signaling pathways, was not always associated with Ras activation. Ras activation also did not correlate with invasion or the expression of proteins associated with tumor cell invasion (estrogen receptor α and cyclooxygenase 2). We then examined the role of Ras signaling in mediating resistance to matrix deprivation-induced apoptosis (anoikis). Surprisingly, we found that ERK and phosphatidylinositol 3-kinase/AKT signaling did not have significant roles in conferring anoikis resistance. Taken together, these observations show that Ras signaling exhibits significant cell context variations and that other effector pathways may be important for Ras-mediated oncogenesis, as well as for anoikis resistance, in breast cancer. Additionally, because ERK and AKT activation are not strictly associated with Ras activation, pharmacological inhibitors of these two signaling pathways may not be the best approach for inhibition of aberrant Ras function in breast cancer treatment.

INTRODUCTION

The three human ras genes (H-, N-, and K-Ras) encode regulated GTP/GDP on-off switches that relay extracellular signals to cytoplasmic signaling networks (1–3). In normal cells, extracellular stimuli that act on diverse cell surface receptors that include receptor tyrosine kinases (e.g., HER2) cause transient activation of Ras. Ras, in turn, associates with and activates multiple downstream effectors that stimulate cytoplasmic signaling cascades that regulate cell proliferation, survival, and differentiation.

The best-characterized downstream effector targets of Ras are the Raf serine/threonine kinases (4). Ras binds to and promotes Raf activation, and activated Raf, in turn, phosphorylates and activates the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK1) and MEK2 dual specificity kinases, which then phosphorylate and activate the ERK1 and ERK2 mitogen-activated protein kinases. The recent identification of mutationally activated B-raf alleles in melanoma, colon, and other types of cancers supports the importance of aberrant Raf->MEK->ERK signaling in Ras-mediated cancer progression and maintenance (5, 6).

The second best characterized Ras effectors are the phosphatidylinositol 3'-kinases (PI3K; Refs. 7, 8). PI3K lipid kinases regulate phosphoinositide lipid metabolism, in particular the conversion of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate. An important consequence of phosphatidylinositol 3,4,5-trisphosphate production is the activation of the AKT serine/threonine kinase. Experimental studies have demonstrated the importance of PI3K/AKT signaling in Ras-induced transformation of some but not all cell types (9–11). The loss of PTEN tumor suppressor function, a negative regulator of PI3K, supports the importance of this signaling pathway in Ras-mediated human cancer development (12).

Aside from Raf and PI3K, other effectors important in promoting Ras transformation include guanine nucleotide exchange factors for the Ras and Rac small GTPases (13–15). Additional Ras effectors have been identified that promote apoptosis (Nore1 and RASSF1), and consequently, antagonize tumorigenesis (8). Altogether, >20 functionally distinct Ras effectors have been identified, and effector utilization by Ras is complex and remains poorly understood. Additional complexity is added by the significant cell context variations observed for Ras effector activation and utilization. For example, although the majority of human pancreatic cancers harbor mutated ras alleles, persistent ERK activation is not associated consistently with Ras activation (16). In a mouse model for pancreatic cancers driven by Ras activation, the tumors that arise possess activated Ras, yet the ERK and AKT pathways are not persistently activated (17). These and other examples emphasize that the signaling outcomes of Ras activation are variable and not always predictable.

The high frequency of ras mutations seen in some neoplasms (e.g., 90% of pancreatic cancers) supports a critical role for aberrant Ras activation in the progression and maintenance of these cancers (18). In contrast, ras mutations are rarely seen in some neoplasms, such as breast cancers (<5%). However, despite this low frequency, there is considerable experimental evidence that aberrant Ras activation and signaling may promote breast cancer development (19). Instead of direct mutational activation in breast cancers, Ras may be activated by persistent upstream signaling. In particular, the HER2 [ErbB2/epidermal growth factor receptor (EGFR)/Neu] receptor tyrosine kinase is overexpressed and persistently activated in approximately 20–25% of human breast cancers (20, 21). Persistent HER2 signaling promotes oncogenic transformation, in part, by activation of Ras (22–24). However, in light of cell context differences in Ras signaling, it is not clear what signaling pathways are stimulated by HER2-induced Ras activation.

One critical step in tumor cell progression is the acquisition of anchorage-independent growth potential (25). Normal breast epithelial cells deprived of matrix attachment undergo apoptosis, or anoikis (26–28), whereas tumor cells or oncogene-transformed cells have escaped this requirement and continue to proliferate (29). The signaling mechanisms that prevent anoikis have been evaluated in experimental model cell systems. For example, Ras activation has been shown to block anoikis in untransformed MCF-10A human breast epithelial...
cells (26). Schulze et al. (27) determined that activation of the Raf/ERK pathway caused induction of an EGFR-mediated autocrine loop that blocked anoikis in MCF-10A cells. Regnato et al. found that EGFR or HER2 activation of ERK was important for blocking anoikis in MCF-10A cells (28). Consistent with the importance of ERK activation, MEK inhibitor treatment to block ERK activation rendered MDA-MB-231 breast carcinoma cells anoikis responsive (30). In contrast, another study found that activation of the PI3K/AKT but not the Raf/ERK pathway in MDCK canine kidney epithelial cells was necessary and sufficient for oncogenic Ras-mediated inhibition of anoikis (31). However, in a third study it was found that oncogenic Ras prevented anoikis in RIE-1 rat intestinal epithelial cells by a mechanism that did not require either of these Ras effector pathways (11). Thus, the importance of specific Ras signaling in preventing anoikis in human breast tumor cells remains to be resolved.

In the present study, we assessed a panel of nine widely studied human breast carcinoma cell lines for the extent of activation of Ras and Ras-mediated signaling pathways and the role of MEK/ERK and PI3K/AKT pathway activation in resistance to anoikis. We found persistent Ras activation in ras mutation-negative breast carcinoma cells and that although ERK and AKT activation was seen in the majority of cell lines, it did not correlate with Ras activation. Furthermore, although cyclooxygenase (COX) 2 expression can be activated by Ras, COX-2 overexpression also did not correlate with Ras activation in the breast cancer cell lines examined. Finally, we found that MEK/ERK and PI3K/AKT activation was not critical for anoikis resistance in breast cancer cell lines. Thus, in breast cancer development, Ras-mediated oncogenesis as well as resistance to anoikis involve signaling mechanisms distinct from those that promote ERK or AKT activation.

MATERIALS AND METHODS

Cell Lines. Immortalized nonmalignant human breast epithelial MCF-10A cells (provided by M. Kinch, Purdue, West Lafayette, IN) were grown in DMEM-F12 supplemented with 5% FCS, 20 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, and 10 μg/ml insulin. All of the human breast carcinoma cell lines were obtained from the American Type Culture Collection and grown in the recommended medium. BT-549 cells were grown in RPMI 1640 supplemented with 10% FCS, and 1 μg/ml insulin. BT-474 cells were grown in RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, 100 mM sodium pyruvate, 2.5 g/liter glucose, and 10 μg/ml insulin. MDA-MB-231 cells were grown in DMEM and 10% FCS. MDA-MB-468 and HS578T cells were grown in DMEM supplemented with 10% FCS and 10 μg/ml insulin. MCF-7 cells were grown in α-MEM supplemented with 10% FCS and 10 μg/ml insulin. T-47D cells were grown in RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, 100 mM sodium pyruvate, 2.5 g/liter glucose, and 7.5 μg/ml insulin. SK-BR-3 cells were grown in McCoy’s 5A and 10% FCS. ZR-75–1 cells were grown in RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, 100 mM sodium pyruvate, and 2.5 g/liter glucose. RIE-1 rat intestinal epithelial cells obtained from Robert J. Coffey (Vanderbilt University, Nashville, TN) were used as controls in the anoikis assays and grown in DMEM supplemented with 5% FCS. All of the growth media were supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin, and all of the cell lines were grown in 5% CO₂ at 37°C.

Protein Expression and Activation. Cells were harvested from 100-mm plates at 80% confluency. Lysates were produced by washing cells with cold PBS and lysed with 100 μl of lysis buffer [20 mM Tris- HCl (pH 7.4), 250 mM NaCl, 0.5% NP40, 3 mM glycerophosphate, 1 mM sodium orthovanadate, and 1× Boehringer Mannheim complete protease inhibitor]. Lysates were clarified by centrifugation at 14,000 × g for 10 min at 4°C and frozen at −80°C when not used immediately. Protein concentration was determined by BCA reaction (Pierce, Rockford, IL). Cell lysates were prepared with 5× Laemmli buffer and boiled for 5 min at 95°C for SDS-PAGE and Western blot analysis.

To determine constitutive phosphorylation status of the EGFR and other family members, immunoprecipitation and Western blotting to detect phosphorylated EGFR or HER2 were performed as described previously (32). Briefly, cells were lysed in normal lysis buffer containing 20 mM HEPES (pH 7.3), 50 mM sodium fluoride, 10% glycerol, 1% Triton X-100, 5 mM EDTA, and 0.5 μM NaCl, supplemented with 1 mM sodium orthovanadate, 6 μg/ml aprotinin, and 10 μg/ml leupeptin, and insoluble fractions removed by centrifugation. Protein concentration was measured (Bio-Rad) and 1 mg of total protein immunoprecipitated with anti-EGFR antibody (Ab22; polyclonal rabbit antiserum raised against recombinant glutathione S-transferase (GST) fusion protein containing the COOH-terminal 100 amino acids of EGFR; ref. 32), anti-HER2 antibody (Ab-1; Neomarkers, Union City, CA), anti-HER3 antibody (Ab1511; rabbit polyclonal antiserum raised against the COOH terminus of HER3), or anti-HER4 antibody (Ab132; rabbit polyclonal antiserum raised against the COOH terminus of HER4) with protein A (A/G) agarose beads (Santa Cruz Biotechnology), then incubated for 2.5 h at 4°C to allow complex formation. Immune complexes were then washed three times with normal lysis buffer, denatured by boiling in 5 min in SDS-PAGE sample buffer, separated on 8% SDS-PAGE, transferred to polyvinylidine difluoride membrane (Bio-Rad), blocked with 3% cold fish gelatin in TBST, then probed overnight with antiphosphotyrosine antibody (PY20; Santa Cruz Biotechnology) in 1% cold fish gelatin in TBST at 4°C, washed with TBST, and incubated with horse-radish peroxidase-conjugated antirabbit or antimouse secondary antibody (Cell Signaling). Signal was detected using enhanced chemiluminescence (Amer sham). Alternately, nonimmunoprecipitated lysates were resolved and transferred as described above and blotted with antireceptor antibody.

For analyses of ERK and AKT activation, total cell lysates (20–40 μg/ lane) were subjected to 8, 10, or 15% SDS-PAGE, and proteins were blotted to polyvinylidine difluoride membrane (Millipore). After boiling in 5% dry milk and TBST, filters were probed with antibodies per the manufacturer’s instructions: Erk (sc93G; Santa Cruz Biotechnology), phospho-AKT, AKT, and phospho-ERK (New England Biolabs, Inc./Cell Signaling Technology).

For Ras, estrogen receptors (ERs), and COX-2 expression, filters were probed with anti-Pan-ras antibody-3 (OP40; Oncogene Research Products), anti-ERα (F-10; Santa Cruz Biotechnology), or anti-COX-2 (Cayman Chemical) antibodies, respectively. Proteins were visualized with peroxidase-coupled secondary antibody using enhanced chemiluminescence (Amersham Pharmacia Biotech). Filters were at times stripped in buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 70°C, washed three times with TBST, blocked, and reprobed with the indicated antibodies.

Ras-GTP Assay. This assay is based on the observation that Ras-GTP, but not Ras-GDP, binds to Raf-1 with high affinity (33) and was done as we have described previously (34). Briefly, bacterially expressed GST fusion protein containing the isolated Ras binding domain of Raf-1 (GST-Raf-RBD) was incubated with 1 μg of total protein of each cell lysate for 30 min at 4°C. The bound GST were collected in centrifugation, washed four times with lysis buffer, resuspended in 5 μl of 5% Laemmli buffer, and boiled for 5 min at 95°C before SDS-PAGE and Western blot analysis using anti-Ras antibody.

Invasion Assays. The transwell Matrigel assay was a modified protocol based on a similar protocol described previously (35). The Matrigel two-dimensional assay, done essentially as described previously (36), provides a qualitative assay that correlates well with in vivo invasion and metastasis. Each cell line was dissociated with enzyme-free cell dissociation medium, and 2 × 10⁵ cells/well in 1 ml of growth medium were plated onto Matrigel-coated dishes. The ability of the cells to invade the Matrigel and organize into honeycomb-like structures was assessed 24 h after incubation using an inverted phase-contrast microscope.

The G8 myoblast assay correlates with the invasive and metastatic ability of aT3 tumors and murine mammary carcinoma cells in vivo (4) and was done as described previously (37, 38). Briefly, G8 myoblasts (obtained from Qin Yu and Ivan Stamenkovic, Harvard Medical School, Boston, MA) were plated at a density of 2 × 10⁵ cells/well in six-well dishes 3 days before the assay began to allow the myoblasts to deposit extracellular matrix proteins on the cell surface. The confluent monolayer was then fixed with DMSO, and a 1-ml suspension containing 2 × 10⁵ cells of each breast carcinoma cell line was added. Invasion was then monitored for 3 days.

Anoikis Assay. We used a DNA fragmentation ELISA to measure anoikis by procedures we have described previously (11). Briefly, cells were plated at 4 × 10⁶ in T175 flasks and grown for 36 h in advance in the growth medium indicated above. Cells were then trypsinized, and 1.5 × 10⁶ cells were plated.
in the presence of growth medium in 60-mm poly(2-hydroxyethyl methacrylate)-coated dishes (39). Pharmacological inhibitors of MEK 1/2 (U0126; provided by James Trzaskos, DuPont) or PI3K (LY294002; AG Scientific) were dissolved in DMSO for use, and their effects were measured relative to DMSO (vehicle)-treated controls. LY294002 (20–50 μM) or U0126 (30 μM) was added to cells after a 30-min incubation in growth medium in suspension. Cells were then harvested at indicated time intervals, washed once with PBS, centrifuged, and frozen at −20°C. Cells were lysed with 1.5 ml of lysis buffer [0.5% Triton X-100, 5 mM Tris (pH 8.0), and 10 mM EDTA] for 30 min on ice. The fragmented DNA-containing soluble fraction was isolated by centrifugation, and apoptosis was quantified by using the Cell Death Detection ELISA kit (Boehringer Mannheim). Samples were assayed in duplicate according to the manufacturer’s instructions. Subsequently, cell lysates for SDS-PAGE and Western blot analysis were generated as described above.

RESULTS

Ras Is Activated in Breast Cancer Cell Lines. Although mutated ras genes are rarely seen in breast cancers, indirect activation of Ras or Ras-mediated signaling pathways may nevertheless be involved in breast cancer development (19). Therefore, we determined whether the levels of activated Ras-GTP were up-regulated periodically in breast carcinoma cell lines that do not harbor mutated ras alleles. For these analyses, we used the immortalized untransformed human MCF-10A cells and nine well-characterized human breast carcinoma cell lines. Of the carcinoma cell lines, only MDA-MB-231 [KRas(G13D)] and Hs578T [H-Ras(Q61L)] are known to be ras mutation positive (5, 40, 41).

Breast carcinoma cell lines obtained from different laboratories or cultured under different conditions may contribute to divergent observations seen with cell lines of the same name, for example, as has been described for MCF-7 strains obtained from different laboratories (42–44). Therefore, for our analyses all of the lines were obtained from the American Type Culture Collection and cultured according to their recommended conditions. Additionally, to ensure that we could accurately compare the different signaling activities and properties of each line, we performed a complete analysis of each line that included some analyses described previously for some cell lines but not others.

For these analyses, we used a pull-down assay using a GST fusion protein containing the isolated Ras-binding domain (GST-Raf-RBD) from Raf-1. The Raf-RBD sequence binds preferentially to activated, GTP-bound Ras protein. All of the cell lines showed variable levels of total Ras proteins, some with multiple bands detected that correspond to the three different Ras proteins recognized by the antibody used (Fig. 1). As expected, MDA-MB-231 cell lines exhibited elevated Ras-GTP levels when compared with levels seen for MCF-10A and other normal human and rodent epithelial cells (11, 45–47). However, although ras mutation-positive, Hs578T cells did not exhibit elevated Ras-GTP levels. Furthermore, four lines that harbor only wild-type ras alleles (BT-549, BT-474, MDA-MB-468, and SK-BR-3) nevertheless exhibited elevated levels of Ras-GTP elevation. Thus, persistent activation of Ras is seen in a majority of breast carcinoma cell lines.

Ras Activation Does Not Correlate with Loss of ERα Expression or Invasive Properties in Vitro. Experimental studies demonstrated that aberrant Ras activation can promote tumor cell invasion and metastasis (48, 49). Therefore, we determined whether Ras activation correlated with the invasive properties of breast carcinoma cells. Additionally, because ERα expression is associated with less aggressive breast cancers and better patient prognosis and survival (50, 51), we also determined whether loss of ERα expression would correlate with Ras activation.

Because different in vitro assays assess distinct aspects of cellular behavior that contributes to invasive activity, we used three in vitro assays to characterize the invasive properties of the breast carcinoma cell lines. First, we determined recently the ability of each cell line to invade through Matrigel and we have summarized those results (45). In agreement with previous studies, we found that the MDA-MB-231, Hs578T, and BT-549, but not the MCF-10A normal or other carcinoma cell lines, showed strong invasive activity (Fig. 2A). Second, we used a two-dimensional Matrigel assay where the cells are allowed to attach and proliferate in contact with basement membrane protein components (52). Previous studies showed that invasive breast carcinoma cells form a lattice under these cell culture conditions, and we observed that the invasive MDA-MB-231, Hs578T, and BT-549 cells all showed this behavior (Fig. 2B). The remaining cell lines failed to form this extensive honeycomb-like appearance growth pattern, although BT-474 showed a limited ability, and MDA-MB-468 cells a greater ability, to form these structures. Third, we assayed the ability of each cell line to invade through a monolayer of G8 myoblasts (37, 38). The G8 monolayer invasion assay provides a simple and highly reproducible test to assay the ability of tumor cells to invade a cell monolayer that is enriched in a variety of extracellular matrix components. Only MDA-MB-231, Hs578T, and BT-549 cells showed the ability to disrupt and degrade the G8 myoblast monolayer, whereas the remaining cell lines attached but showed limited proliferation and growth into the G8 monolayer (Fig. 2C). Thus, we found that the three assays indicated consistently that MDA-MB-231, Hs578T, and BT-549 cells exhibit invasive properties in vitro. However, MDA-MB-231 and SK-BR-3, but not Hs578T, cells showed elevated Ras-GTP levels. Furthermore, although BT-474, SK-BR-3, and MDA-MB-468 showed elevated Ras-GTP levels, they exhibited little invasive potential. Thus, Ras activation alone is not sufficient to promote the invasive properties of breast carcinoma cells.

Western blot analyses showed that the BT-474, MCF-7, and T-47D cell lines were positive for ERα protein expression (Fig. 1). In addition, MCF-10A and ZR-75-1 were weakly positive for ERα expression. The remaining cell lines were ERα negative. These results are consistent with the prior analyses of ERα mRNA (53) and protein expression (54). The four ERα-positive lines are noninvasive, whereas the three invasive lines are negative for ERα expression. However, although MDA-MB-468 and SK-BR-3 cells lack ERα expression, neither exhibited significant invasion activity in vitro. Thus, there is an incomplete correlation between loss of ERα expression and increased invasive potential in breast carcinoma cell lines. 

HER2 Expression May Contribute to Ras Activation. One mechanism by which Ras may become activated in ras mutation-
negative tumor cells is by aberrant activation of HER2/Neu/ErbB2 and related receptor tyrosine kinases (22–24). Therefore, we determined the level of expression and activation of the four HER family receptor tyrosine kinases. In agreement with previous studies, we found increased autophosphorylation and activation of HER2 (Fig. 3) in SKBR-3, BT-549, and ZR-75-1 cells, and consequently, may contribute to the elevated Ras-GTP levels seen in BT-549 and SKBR-3. However, ZR-75-1 cells also showed elevated HER2 activation, yet this line did not display elevated Ras-GTP levels. In contrast, no significant constitutive phosphorylation of any HER family protein was seen for MDA-MB-468 cells, despite known EGFR mRNA overexpression (53), yet these ras mutation-negative cells did display elevated Ras-GTP levels. Thus, no clear correlation between HER overexpression, activation, and Ras-GTP elevation was seen.

**ERK and AKT Activation Does Not Correlate with Ras Activation.** We next wanted to determine whether two key Ras effector signaling pathways are persistently activated in breast carcinoma cell lines where Ras-GTP levels are persistently up-regulated. To assess the Raf/MEK/ERK signaling pathway, the levels of phosphorylated and activated p44 ERK1 and p42 ERK2 were determined. All of the cell lines showed equal amounts of total ERK1 and ERK2 protein (Fig. 4A). However, four of the nine breast carcinoma cell lines (BT-474, MDAMB-231, MDA-MB-468, and T-47D) showed levels of activated ERK that were significantly above that seen for the control untransformed MCF-10A cells (Fig. 4A) and other normal human and rodent epithelial cells (11, 45–47). Three of these four cell lines also exhibited elevated Ras-GTP levels. However, T-47D cells showed elevated ERK activation, yet these cells do not possess mutated Ras or overexpressed HER2 nor do they demonstrate Ras activation (Fig. 1), indicating that ERK is activated by a Ras-independent mechanism in these cells. In contrast, although Hs578T cells are ras mutation-positive, no significant ERK activation was seen. Thus, ERK activation does not correlate absolutely with the presence of mutated ras or Ras activation.

We next assessed activation of PI3K by evaluating the levels of total and phosphorylated, activated AKT. Although the same cell lysates showed equivalent expression of total ERK, we found significant variation in the levels of expression of total AKT (Fig. 4B) with
highest levels seen in MCF-10A, MCF-7, and BT-474 cells. The level seen for MCF-10A cells is comparable or higher than the level we have seen for other normal human and rodent epithelial cells (11, 45–47). Similarly, the levels of phosphorylated and activated AKT levels were also highly variable, with highest activity seen for Hs578T, MDA-MB-468, and BT-474. Because Hs578T cells did not show elevated Ras-GTP or HER activity, AKT activation must occur by a different mechanism (55). Surprisingly, although MDA-MB-231 cells harbor mutated ras, no significant AKT activation was seen. Thus, AKT activation did not correlate strongly with Ras activation.

**COX-2 Expression Does Not Correlate with Ras Activation.**

Overexpression of COX-2, an inducible enzyme that catalyzes prostaglandin biosynthesis, has been implicated in breast tumor cell invasion (56, 57). Furthermore, previous studies determined that COX-2 expression is up-regulated by HER2-mediated transformation of human breast epithelial cells, as a consequence of activation of the Ras/ERK cascade, and correlates with HER2-overexpressing breast cancers (58). Ras-mediated transformation of fibroblasts and epithelial cells also causes ERK-dependent up-regulation of COX-2 (59). Therefore, we determined whether COX-2 up-regulation correlates with Ras activation by performing Western blot analysis to determine the expression level of total COX-2 protein. We found that COX-2 expression levels were variable, with highest levels seen in MCF-10A, BT-549, T-47D, and Hs578T cells (Fig. 5). Thus, COX-2 overexpression correlated neither with HER2 or Ras activation nor with invasive potential.

**ERK or AKT Activation Is Not Required for Breast Carcinoma Cell Resistance to Anoikis.** Studies evaluating breast cell lines transformed by ectopic overexpression of activated HER2 or Ras provides support for both the Raf/ERK and PI3K/AKT cascades in preventing anoikis (26–28). Therefore, we evaluated the importance of Ras, ERK, and AKT activation in the anoikis resistance phenotype of breast carcinoma cells that harbor endogenously activated HER2 or Ras.

We determined previously that RIE-1 rat intestinal epithelial cells are highly sensitive to anoikis and that Ras activation blocked this sensitivity (11). We evaluated the untransformed MCF-10A cells and five ERK and/or AKT activation-positive human breast carcinoma cell lines to determine their sensitivity or resistance to anoikis. Similar to RIE-1 cells, we found that MCF-10A cells were sensitive to anoikis, which is consistent with observations from previous studies (26–28). We also found that MDA-MB-468 cells were surprisingly sensitive to anoikis, whereas Hs578T were moderately resistant, and the MDA-MB-231, BT-474, and T-47D cell lines were strongly resistant to anoikis (Fig. 6; data not shown).

To determine whether the Raf/MEK/ERK pathway was necessary for anoikis resistance, we examined the three cell lines that were resistant to anoikis and possessed elevated levels of activated ERK; MDA-MB-231, BT-474, and T-47D. We first verified that their activated ERK levels remained elevated when the cells were grown in suspension, and we determined that treatment with the MEK1/2-specific inhibitor UO126 effectively reduced ERK activity in each cell line (Fig. 6). Surprisingly, no significant increase in apoptosis was seen upon ERK inhibition. Thus, ERK activation was not necessary to confer resistance to anoikis.

Of the three anoikis-resistant cell lines, only BT-474 and T-47D cells showed elevated AKT activation (Fig. 4B). Therefore, elevated AKT activation is not required for the anoikis resistance of MDA-MB-231 cells. To evaluate the contribution of the PI3K/AKT signaling pathway in promoting resistance to anoikis for BT-474 and T-47D cells, we used the LY29004 P33K inhibitor. We first verified that their activated AKT levels were unchanged when the cells were grown in suspension, and we then determined the effective concentration for
LY294992 inhibition of AKT activation for each cell line. Whereas 20 μM LY294002 treatment was effective in blocking AKT activation in T-47D and Hs578T cells, there was only minimal inhibition of AKT activation in BT-474 cells when treated with up to 100 μM LY294002 (Fig. 7). This suggests that activation of AKT in this cell line is through a different mechanism other than PI3K activation.

At high concentrations of LY29004, nonspecific inhibitory activities may be seen (60). Therefore, we only evaluated the importance of AKT activation for the anoikis resistance of T-47D cells. Surprisingly, inhibition of AKT activity did not render T-47D cells sensitive to anoikis. Treatment with 50 μM LY294002, which caused additional AKT inhibition, also did not cause an increase in apoptosis (data not shown). Thus, AKT activation is not necessary for the anoikis-resistant phenotype of T-47D cells.

**DISCUSSION**

Experimental studies using model cell systems transformed by ectopic overexpression of mutant Ras have established the importance of ERK and AKT activation in promoting Ras-mediated oncogenesis (1–3). Similarly, ectopic overexpression of activated HER2/Neu has been shown to cause transformation by a Ras-dependent mechanism (22–24). However, whether aberrant activation of endogenous HER2 and Ras promotes the same signaling consequences and whether these signaling activities are important for maintenance of the tumorigenic state in human tumor cells has not been adequately explored. The goal of this study was to provide a characterization of the involvement of Ras activation and signaling in the invasive growth properties of breast cancer cells (Table 1). First, we found that Ras is chronically activated in breast carcinoma cells that lack mutated ras. Second, Ras

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<th>Table 1</th>
<th>Properties of human breast carcinoma cell lines</th>
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\( ^{\alpha} \) Quantitation determined by visual determination of data from Figs. 1–4. 
\( ^{\beta} \) ER, estrogen receptor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; −, no detectable band; +, faint detectable band; ++, clearly detectable band; ++++, strongly detectable band.
\( ^{\beta} \) Invasion determined by Matrigel and G8 assays.
\( ^{d} \) ras mutation positive.
activation was not always associated with persistent activation of ERK or AKT. Third, Ras activation did not correlate with either the invasive capacity of breast carcinoma cells or the up-regulation of COX-2. Finally, in contrast to studies with model cell culture systems, we found that ERK and AKT activation are not critical for the ability of human breast carcinoma cells to escape matrix deprivation-induced apoptosis. Taken together, these results highlight the complex nature of Ras signaling and show that signaling pathways are not simply linear and that Ras exhibits cell context differences in promoting breast carcinoma growth.

Although mutated ras genes are not associated with the majority of breast cancers, we found significant levels of activated Ras in five of nine breast carcinoma cell lines. Thus, aberrant Ras activation may be a common feature of breast cancers. Elevated activation of HER2 and related receptors may contribute to Ras activation in ras mutation-negative breast cancers. However, in contrast to cells transformed by ectopic expression of HER2/Neu (22, 23), endogenous HER2 activation alone may not be sufficient to cause Ras activation, for example, in the HER2-positive ZR-75–1 cells that did not show elevated Ras activity. Our results are in general agreement with those of VonLintig et al. (24), who found that HER2 overexpression correlated with increased Ras-GTP formation in breast tumor tissue and cell lines.

Ectopic overexpression of mutated Ras promotes persistent activation of ERK and AKT in NIH 3T3 and other cell types (2, 3). Therefore, based on these studies it was unexpected that ERK and AKT activation was not associated strictly with Ras activation. However, when studied in cells that harbor endogenous Ras activation, ERK and AKT activation is not consistently associated with expression of mutated Ras (16, 17). Taken together, these observations emphasize that Ras signaling cascades are not simply linear and that Ras signaling may exhibit significant cell context variations (46). Therefore, ras mutation status may not be a reliable indicator of patients who would benefit from treatment with pharmacological inhibitors of these two signaling pathways.

The acquisition of resistance to matrix deprivation-induced apoptosis is a crucial step that promotes the anchorage-independent growth of cancer cells (29). Expression of mutated Ras renders a wide variety of cell types resistant to anoikis. For MDCK cells, AKT rather than ERK activation was shown to be necessary and sufficient to promote Ras inhibition of anoikis (31). However, when evaluated in MCF-10A cells, Ras activation of the Raf/ERK cascade alone was sufficient to block anokis (27). Similarly, HER2-mediated transformation of MCF-10A cells prevented anoikis by activation of ERK (28). Hence, activation of AKT or of ERK represent two mechanisms by which Ras may prevent anoikis. However, unexpectedly, our analyses determined that neither ERK nor AKT activation was critical for the anoikis resistance of breast carcinoma cells. These results are similar to those we observed for Ras-transformed RIE-1 cells, where neither ERK nor AKT activation were necessary for Ras inhibition of anoikis (11). Therefore, the signaling pathways responsible for the anoikis inhibition in these breast carcinoma cell lines remain to be elucidated.

In summary, our studies provide additional evidence that the signaling and biological consequences of Ras will vary significantly with cell context. Thus, whereas model cell systems such as NIH 3T3 cells have provided powerful models for delineating the signaling pathways that are activated by Ras and for implicating specific signaling pathways in Ras-mediated oncosign, these observations cannot be extrapolated easily to human tumor cells. These observations also account for the limited clinical effectiveness of signal transduction molecule-based therapies currently undergoing clinical evaluation (3, 61). Hence, an important focus for future studies will be to determine whether other Ras effector signaling pathways, for example the RAF/GEF/Ral (47) or the Tiam1/Rac pathway (14, 15), are more critically linked to aberrant Ras activation in human cancers.

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