Abstract
Receptor tyrosine kinases of the ErbB family are implicated in a number of cancers, including that of the breast. ErbB receptors are activated by ligand-induced formation of homodimers and heterodimers. Receptor heterodimerization is thought to play a critical role in breast cancers overexpressing multiple members of the ErbB family. Although coexpression of ErbB receptors is associated with poor patient prognosis, the mechanisms by which receptor heterodimerization regulates tumor progression are not clear, due in part to a lack of methods that allow controlled activation of specific receptor heterodimers in mammary epithelial cells. Here, we report an approach to activate ErbB1-ErbB2 heterodimers in a nontumorigenic breast epithelial cell line, MCF-10A, without interference from endogenous ErbB receptors. Using such a method, we show that whereas both ErbB2 homodimers and ErbB1-ErbB2 heterodimers were equally potent in activating the Ras/mitogen-activated protein kinase pathway, the heterodimers were more potent in activating the phosphoinositide 3’-kinase (PI3K) and phospholipase Cγ1 pathways than ErbB2 homodimers. We combined the dimerization system with a three-dimensional cell culture approach to show that whereas both ErbB2 homodimers and ErbB1-ErbB2 heterodimers induced disruption of three-dimensional acini-like structures, only heterodimers promoted invasion of cells through extracellular matrix. The ability of heterodimers to induce invasion required the ErbB1 kinase activity and required activation of PI3K, Ras/mitogen-activated protein kinase, and phospholipase Cγ1 signaling pathways. Thus, we have identified cell invasion as a heterodimer-specific biological outcome and suggest that coexpression of ErbB1 may critically regulate invasive progression of ErbB2-positive breast cancers. (Cancer Res 2006; 66(10): 5201-8)

Introduction
The epidermal growth factor (EGF) receptor (EGFR) family of receptor tyrosine kinases consists of four members, ErbB1/EGFR/HER1, ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4, herein called the ErbB family. The ErbB receptors are activated by at least 12 different ligands, including EGF, transforming growth factor-α (TGF-α), and neuregulins. Ligand stimulation induces both homodimerization and heterodimerization among the ErbB family members that results in receptor transphosphorylation and activation of downstream signaling. Altered expression of EGF ligands and ErbB receptors is implicated in several carcinomas. In breast, amplification and overexpression of ErbB2 occurs in 25% to 30% of cancers and is associated with comedo-type ductal carcinoma in situ and poor clinical prognosis in node-positive patients (for review, see refs. 1, 2).

Among the other members of ErbB family, ErbB1 expression is observed in >50% of breast cancers and correlates with estrogen receptor–negative status, poor tumor grade, and aneuploidy (3). However, it is not clear whether ErbB1 has a strong oncogenic role in mammary epithelia. For instance, overexpression of ErbB1 in the mouse mammary gland induces infrequent adenocarcinomas, which contrasts with the ability of ErbB2 overexpression to induce frequent mammary tumors with relatively short latency (4, 5). In addition, we have shown that activation of ErbB2 homodimers, but not activation of ErbB1 homodimers, induces transformation of three-dimensional organized breast epithelial acini-like structures (6). Investigations using fibroblasts also show that ErbB1 is almost 100-fold weaker than ErbB2 in its transforming ability (7), suggesting that ErbB1 may not be a strong oncogene in many cell types, including mammary epithelia.

Even if ErbB1 is not a strong oncogene by itself, several lines of evidence suggest that it plays a critical role during initiation and progression of ErbB2-positive breast cancers. Early indications came from studies using fibroblasts that show a synergistic interaction between ErbB1 and ErbB2 during in vitro transformation (8). Later studies using mouse models show that ErbB1 levels are up-regulated in mammary tumors induced by overexpression of ErbB2/Neu, and coexpression of ErbB2/Neu and TGF-α (an ErbB1 ligand) show synergistic effects by inducing development of multifocal mammary tumors with short latency (9). Furthermore, inhibition of ErbB1 using AG1478, an ErbB1 kinase inhibitor, in mice coexpressing ErbB2/Neu and TGF-α significantly delays tumor formation (10), suggesting that ErbB1 and ErbB2 cooperate during mammary tumorigenesis in vivo. A cooperating role for ErbB1 in human breast cancer is provided by a recent clinical study that shows that coexpression of ErbB1 and ErbB2 correlates with the presence of tyrosine phosphorylated ErbB2 and short patient survival (11), squarely implicating ErbB1 and ErbB2 coexpression with receptor activation and poor patient prognosis.

Although we do not fully understand how ErbB1/ErbB2 heterodimers promote carcinoma, several mechanisms have been proposed. Heterodimerization can promote increased affinity for ligand binding or can interfere with endocytosis and downregulation of ErbB1 receptors, thus resulting in prolonged activation of downstream signaling (2). Heterodimerization can also promote phosphorylation of novel tyrosine residues in the...
cytoplasmic domain of ErbB receptors (12). The consequence of these heterodimer-specific phosphorylation events is not known and it is also not clear if there are heterodimer-specific biological processes that regulate transformation of mammary epithelial cells.

Most of the studies aimed at investigating signaling and biological outcomes downstream of ErbB heterodimers use either hematopoietic cells that lack expression of endogenous ErbB receptors or fibroblasts that express very low levels of the receptors (13). These cell systems, albeit being extremely useful, do not represent the target tissues that are transformed by oncogenic ErbB signaling and hence suffer from lack of in vivo significance. In addition, EGF stimulation of cells expressing both ErbB1 and ErbB2 will result in formation of both ErbB1-ErbB1 homodimers and ErbB1-ErbB2 heterodimers and thus confound the ability to identify heterodimer-specific outcomes. One approach that has provided most insight into the role of ErbB2-containing heterodimers in breast tumor–derived cells is using single-chain antibody-mediated inhibition of ErbB2 function (14). These studies show that ErbB2 plays a critical role in ligand-induced activation of downstream signaling molecules, proliferation, and invasion (15, 16). However, this system is best suited for reverse approaches, where the role of an ErbB receptor dimer is deduced by inactivating it. There are no forward approaches available to inducibly activate specific ErbB receptor heterodimers and investigate how different ErbB dimers regulate initiation and progression of breast cancer.

To this end, we exploit a novel approach, originally reported by Crabtree and Schreiber laboratories, to control dimerization and activation of signaling molecules such as the Src family of kinases using a dimeric version of the immunosuppressive drug FK506, called FK1012, and its binding protein FK506-binding protein (FKBP; ref. 17). We first reported a modified version of this approach to control dimerization and activation of ErbB receptor homodimers in fibroblasts and in human mammary epithelial cell line MCF-10A (6, 18). Activation of ErbB2 homodimers induces growth factor–independent proliferation and promotes disruption of three-dimensional mammary acini-like structures to form abnormal, noninvasive lesions (6). Here, we show that in addition to regulating homodimerization of ErbB receptors, we can engineer cells to induce formation of specific ErbB receptor heterodimers. This approach allows us to specifically activate either ErbB2-ErbB2 homodimers or ErbB1-ErbB2 heterodimers within the same cell by choosing different synthetic dimerizing ligands. Using such a system, we show that ErbB1/ErbB2 heterodimers promote activation of dimer-specific signaling pathways and identify invasive behavior as a biological process that is downstream of ErbB1-ErbB2 heterodimers and not ErbB2 homodimers. This study provides a novel insight into biological mechanisms that are critical in breast cancers coexpressing multiple ErbB receptors.

Materials and Methods

Plasmids and reagents. The retroviral vector encoding p75.ErbB2.FKB-P.HA chimera is described elsewhere (18). The p75.ErbB1.FRB.Glu-Glu was generated by replacing the FKBP.HA portion of the p75.FKBP.HA with FRB and adding a Glu-Glu epitope tag in pBabe.puro retroviral backbone. Chemical inhibitors AG1478, U0126, LY294002, U73122, and U73343 were purchased from Calbiochem (San Diego, CA), Antibodies against hemagglutinin (HA) and Glu-Glu (Covance, Berkeley, CA), phosphotyrosine and phospho–extracellular signal-regulated kinase 2 (ERK2; BD Transduction Laboratories, San Diego, CA), β-actin (Sigma, St. Louis, MO), Laminin V (Chemicon, Temecula, CA), p75 antibody (Neomarkers, Fremont, CA), Akt, phospho-Akt (S472), and phospho–phospholipase Cγ1 (PLCγ1, Tyr735) and PLCγ1 antibody from Cell Signaling (Beverly, MA). AP1510 and rapamycin analogues (rapalog) were obtained from ARIAD Pharmaceuticals (Cambridge, MA). Protein assay reagent was from Bio-Rad Laboratories (Hercules, CA), protein G-coupled Sepharose beads were from Amersham Pharmacia Biotech (Piscataway, NJ). Matrigel was from BD Transduction Laboratories, and Vitrogen Collagen was from Angiotech Biomaterials (York, United Kingdom).

MCF-10A cells and three-dimensional cell culture. MCF-10A cells coexpressing ErbB2-FKBP and ErbB1-FRB chimeras were generated by infection of MCF-10A.ErbB2 cells with retrovirus expressing p75.B1-FRB as outlined previously (6, 19). For generation of three-dimensional acini, MCF-10A cells were cultured on Matrigel in eight-chamber RS glass slide as described before (6, 19). To monitor the effect on different dimers to induce three-dimensional proliferation, the structures were allowed to grow for 6 days and stimulated with either 1 mmol/L AP1510 or 200 mmol/L rapalog for 48 hours.

Invasion assays. The Matrigel Collagen mix was generated by mixing 1.0 mL Matrigel, 1.0 mL Vitrogen Collagen 100×125 μL of 10× PBS: 125 μL NaOH, and 25 μL of 0.1 mol/L HCl, and layered on eight-chamber slides as described above. The final collagen concentration was 1 to 1.5 μg/mL. Anti–Laminin V immunofluorescence was done as described previously (19).

All quantitative analyses of invasion were done using BD Biocat Matrigel invasion chambers (BD Biosciences, San Diego, CA). Briefly, 1 × 10⁶ cells in 1.5 mL medium were added into upper chamber of six-well dishes, and 2.0 mL medium was added to the lower chamber in the presence of absence of rapalog. Cells were incubated at 37°C for 24 hours, then fixed in 10% (w/v) buffered formalin, and stained with 2% Giemsa (Life Technologies, Carlsbad, CA) in Gurr solution. Cells on the upper surface of chambers were removed with cotton swabs and cells that invaded the lower surface were counted.

Immunoblot analysis and immunoprecipitation. MCF10A.ErbB2 or MCF10A.ErbB2/B1 cells were plated at a density 1 × 10⁶ per well in a 10 cm culture dish. After 48 hours, the cells were switched to serum-free medium for 24 hours and subsequently stimulated with indicated amounts of AP1510 and rapalog for 30 minutes. Cells were rinsed with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer (10 mmol/L Tris (pH 7.2), 158 mmol/L NaCl, 1 mmol/L EDTA, 0.1% Triton X-100, 1 mmol/L Na₂VO₃, 1 mmol/L phenylmethylsulfonyl fluoride, 100 units/ml aprotinin, 10 μg/mL leupeptin, and 10 μg/mL pepstatin), centrifuged at 15,000 × g for 15 minutes at 4°C. The lysates were analyzed by immunoblotting or immunoprecipitation as described previously (18).

Fluorescence-activated cell sorting analysis. MCF10A.ErbB2/B1 cells were plated at a density 1 × 10⁶ per well in a 10 cm culture dish. After 48 hours, the cells were switched to serum-free medium for 24 hours and subsequently stimulated with indicated amounts of AP1510 and rapalog for 18 hours. Cells were trypsinized, resuspended in 10% serum-containing medium, and washed with 1× PBS. The cells pellet was resuspended in 0.5 mL of 1× PBS and fixed overnight in ice-cold ethanol. The fixed cells were washed in 1× PBS and stained with 10 μg/mL propidium iodide and analyzed by fluorescence-activated cell sorting (FACS). The data were used to calculate the percentage of cells in the S phase of the cell cycle.

Results

Receptor chimera and heterodimerization. To develop a controlled dimerization system that will allow us to specifically activate ErbB receptor heterodimers, we adopted a method developed by ARIAD Pharmaceuticals that uses the ability of rapamycin to promote heterodimerization between proteins fused to FKBP and FK506 FKBP associated protein (FRAP; refs. 20, 21). To circumvent the growth-suppressive properties of rapamycin, rapalogs that lack the ability to bind endogenous FRAP or its ability to inhibit mammalian target of rapamycin signaling were generated (20, 21). Rapalogs, however, bind to mutant version of FRAP (T2098L), identified by phage-display mutagenesis screen, and thus promote heterodimerization between proteins fused to FKBP and T2098L-FRAP (20, 21).

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We generated an ErbB1 chimera by fusing a 93-amino-acid domain of T2098L-FRAP, called FRB, to the cytoplasmic domain of ErbB1. The extracellular domain of ErbB1 was removed and replaced with the extracellular domain of p75, the low-affinity nerve growth factor receptor, to prevent binding to natural EGF family of ligands. A COOH-terminal Glu-Glu epitope tag was added to facilitate biochemical analyses (Fig. 1A). Retrovirus encoding the ErbB1 chimera was used to infect MCF-10A cells that were previously engineered to express chimeric ErbB2 with a FKBP domain in its COOH termini (Fig. 1I; ref. 6). The resulting cells, 10A.ErbB2/ErbB1, can be stimulated with either AP1510 to promote homodimerization of ErbB2-FKBP fusions or rapalog to induce heterodimerization between ErbB2-FKBP and ErbB1-FRB chimeras (Fig. 1B and C).

Cell lysates from MCF-10A cells expressing different chimeras were used to determine the expression of ErbB2 or ErbB1 chimera and the ratio of ErbB1 versus ErbB2 chimera. Both ErbB1 and ErbB2 chimeras were expressed at detectable levels as monitored using Glu-Glu or HA epitope tags, respectively (Fig. 1D). The ErbB1 and ErbB2 chimeras in 10A.ErbB2/ErbB1 were expressed at comparable levels as determined using antibodies against the p75 domain common to both chimeras (Fig. 1D).

**Synthetic ligand–induced activation of receptor heterodimers.** We have previously shown that AP1510 induces homodimerization and activation of ErbB2-FKBP chimera expressed in 10A.ErbB2 cells (6). To rule out the possibility that rapalog stimulation has nonspecific effects on FKBP-fused ErbB2 chimera, we monitored the effect of stimulating 10A.ErbB2 cells with rapalog. Rapalog did not induce any increase in the basal phosphorylation of the ErbB2 chimera observed in 10A.ErbB2 cells, demonstrating that the ErbB2-FKBP chimera did not nonspecifically respond to the heterodimerizing drug, rapalog (Fig. 2A).

To determine whether we can control activation of ErbB2 homodimers and ErbB1-ErbB2 heterodimers by choosing appropriate small-molecule ligands, 10A.ErbB2/ErbB1 cells were stimulated with AP1510 or rapalog. Using the phosphorylation status of ErbB2 as a measure of activation, we determined that stimulation with either AP1510 or rapalog resulted in phosphorylation of the ErbB2 chimera (Fig. 2B). The ability of AP1510 to induce phosphorylation of receptor is consistent with our previous results demonstrating AP1510-induced formation of ErbB2-FKBP-ErbB2-FKBP homodimers (18). Because the rapalog does not have the ability to induce homodimerization of FKBP-fused proteins, the rapalog-induced phosphorylation of ErbB2-FKBP chimera observed in 10A.ErbB2/ErbB1 cells suggests that rapalog induces activation of ErbB2 in an ErbB1-ErbB2 heterodimer context.

To test whether rapalog-induced ErbB1-ErbB2 dimers can be detected as a biochemical complex, we did coimmunoprecipitation analyses. ErbB2 or ErbB1 was immunoprecipitated from cell lysates derived from cells treated with either AP1510 or rapalog and analyzed for the presence of the heterodimerization partner. Stimulation with AP1510 did not result in coimmunoprecipitation of ErbB1 and ErbB2, whereas stimulation with rapalog resulted in a physical complex between ErbB1 and ErbB2 (Fig. 2B and C) demonstrating that rapamycin induces formation of

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**Figure 1.** Receptor chimeras and expression in MCF-10A. A and B, cartoon of the chimeric receptors and the synthetic dimerizing ligands. C, a cartoon of ligand-induced dimers. D, expression of the chimeric receptors in MCF-10A cells (10A), MCF-10A cells expressing ErbB2 alone (10A.ErbB2), or cells expressing both ErbB1 and ErbB2 chimera (10A. ErbB2/B1) was analyzed by immunoblotting with antibodies against epitope tags (HA or Glu-glu) or with anti-p75 antibodies. The immunoblot was reprobed with anti-β-actin to control for equal loading.

**Figure 2.** Rapalog-induced heterodimerization. A, cells expressing ErbB2 only (10A.ErbB2) were stimulated with rapalog for 30 minutes and lysates were analyzed with anti-pTyr antibodies. The immunoblot was reprobed with anti-β-actin to control for equal loading. B, cells expressing both ErbB1 and ErbB2 chimera (10A.ErbB2/B1) were stimulated with either homodimerizer (AP1510) or heterodimerizer (rapalog) and the cell lysates were used to immunoprecipitate ErbB2 (IP:HA). The ErbB2 immunoprecipitates were immunoblotted using anti-pTyr (IP:pY) or anti-Glu-Glu antibodies. The immunoblot was reprobed with anti-β-actin to control for immunoprecipitation. C, ErbB1 was immunoprecipitated from the lysates in (B) using Glu-Glu antibodies and immunoblotted for ErbB2 (IP:HA). The immunoblot was reprobed with anti-Glu-Glu to control for immunoprecipitation. D, 10A.ErbB2/B1 cells were stimulated with increasing concentrations of rapalog and ErbB1 was immunoprecipitated (IP-Glu-Glu) and immunoblotted for anti-pTyr (IP:pY).
ErbB1-ErbB2 heterodimers. We also determined that 200 nmol/L rapalog induced maximal stimulation of tyrosine phosphorylation (Fig. 2D). Thus, rapalog stimulation induces dimerization and phosphorylation in a dose-dependent manner.

**Rapalog-induced heterodimers activate binding of downstream effectors and signaling.** We have previously shown that AP1510-induced activation of ErbB1 or ErbB2 homodimers results in recruitment of Src homology (SH2) domain-containing proteins such as growth factor receptor binding protein 2, Shc, and Cbl (18). To determine whether rapalog-induced heterodimers associate with SH2 domain–containing proteins, we determined the ability of PLCγ1 to associate with ErbB chimera. We chose to investigate PLCγ1 because previous studies have shown that among the ErbB receptors, PLCγ1 binds to ErbB1 with high affinity (22). PLCγ1 was coimmunoprecipitated with ErbB1 and ErbB2 only when cells were stimulated with rapalog (Fig. 3A and B) and was not observed in a complex with ErbB2 when ErbB2 was activated as a homodimer (Fig. 3B, AP1510 lane). This observation suggested that PLCγ1 is selectively recruited to ErbB1-ErbB2 heterodimer (Fig. 3B, rapalog lane) to form an ErbB2-ErbB1-PLCγ1 trimeric complex. To investigate whether the ability of ErbB1-ErbB2 to associate with PLCγ1 correlates with its ability to activate PLCγ1, we determined whether ErbB2 homodimers and ErbB1 homodimers differ in their ability to promote phosphorylation of PLCγ1. Because previous studies have shown that phosphorylation of PLCγ1 at Tyr771 correlates with activation (22), we monitored changes in phosphorylation of PLCγ1 using an antibody that recognizes phosphorylated Tyr771 on PLCγ1. ErbB1-ErbB2 heterodimerization was significantly more potent in inducing phosphorylation of PLCγ1 (Fig. 3C), suggesting that in addition to associating with PLCγ1, ErbB1-ErbB2 heterodimers were more potent that ErbB2 homodimers in activating signaling by PLCγ1. The results also suggest that rapalog-induced heterodimers are functionally active in recruiting and activating specific cytoplasmic signaling molecules.

We have previously shown that activation of ErbB1 or ErbB2 homodimers in both Rat1 fibroblasts and MCF-10A cells results in stimulation of ERK phosphorylation (6), demonstrating that controlled homodimerization can activate downstream signaling. To determine whether the rapalog-induced heterodimerization activated downstream signaling, lysates from cells stimulated with AP1510 or rapalog were analyzed for phosphorylation of ERK and Akt. Both AP1510 and rapalog stimulation induced phosphorylation of ERK (Fig. 3A), suggesting that synthetic ligand-induced homodimers and heterodimers activate the Ras/mitogen-activated protein (MAP) kinase (MAPK) pathway at similar levels. In contrast, activation of ErbB2-ErbB1 heterodimer was more potent in its ability to phosphorylate Akt than ErbB2-ErbB2 homodimer, suggesting that heterodimers are potent activators of the phosphoinositide 3′-kinase (PI3K) pathway (Fig. 3A), consistent with several previous studies that show phosphorylation of Akt is a downstream target of EGF stimulation. These observations show that controlled heterodimerization activates signaling pathways downstream of ErbB signaling.

**ErbB1-ErbB2 heterodimers potently activate proliferation of cells on standard culture plastic and three-dimensional culture.** We have previously shown that activation of either ErbB1 or ErbB2 homodimers induces EGF-independent proliferation of MCF-10A cells (6). To determine whether controlled activation of receptor heterodimers also promotes EGF-independent proliferation, we determined the ability of MCF-10A cells expressing ErbB1 or ErbB2 chimera to proliferate in the absence of EGF. Both rapalog and AP1510 stimulation induced EGF-independent proliferation of MCF-10A cells (Fig. 4A), whereas ErbB2-ErbB1 heterodimers were almost 2-fold more potent than ErbB2 homodimers in their ability to induce EGF-independent proliferation of MCF-10A cells (Fig. 4A).

Although proliferation of cells on plastic dishes is an excellent indicator of oncogenic potential in vivo, we have previously shown that although both ErbB1 and ErbB2 homodimers can induce EGF-independent proliferation of MCF-10A cells grown on plastic dishes, they differ in their ability to disrupt three-dimensional organized acini-like structures grown on a bed of extracellular matrix (Matrigel; ref. 6). Activation of ErbB2 homodimers, but not activation of ErbB1 homodimers, induced proliferation and disruption of growth-arrested three-dimensional acinar structures (6). These results, and others (23), highlight the importance of studying oncogenesis in three-dimensional organized epithelial structures. We investigated whether activation of ErbB1-ErbB2 heterodimers and ErbB2 homodimers differ in their ability to disrupt three-dimensional acini. Consistent with our previous observations, activation of ErbB2 homodimers resulted in disruption of 30% of acini, as determined by the presence of multicellular structures (see Fig. 4B for details). Activation of ErbB2-ErbB1 heterodimers resulted in disruption of almost 60% of the structures, suggesting that the heterodimers were almost 2-fold more potent in disrupting organized three-dimensional acini. Thus, although ErbB1 homodimers lack the ability to affect MCF-10A three-dimensional acini (6), coexpression of ErbB1 significantly potentiate the ability of ErbB2 to deregulate proliferation control in vivo.

**Heterodimers induce invasion.** Neither ErbB1 homodimers nor ErbB2 homodimers have the ability to promote invasion of MCF-10A cells (6). Because overexpression of ErbB2 in breast cancer cell lines is thought correlate with acquisition of invasive properties (16, 24), we determined whether activation of ErbB2

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**Figure 3.** Heterodimer-induced activation of downstream signaling. 10A.ErbB2/B1 cells were stimulated with rapalog or AP1510 and the lysates were used to immunoprecipitate ErbB1 (A) or ErbB2 (B) chimera and immunoblotted with anti-PLCγ antibodies. 10A.ErbB2/B1 cells were stimulated with rapalog or AP1510 for the indicated lengths of time. Lysates were analyzed with antibodies that recognize phosphorylated forms of PLCγ1 (C), ERK (p-ERK1/2) or Akt (p-Akt), or total ERK (ERK2) or Akt (D).
homodimers or ErbB1-ErbB2 heterodimers promote formation invasive structures. Activation of heterodimers, and not activation of homodimers, resulted in formation of small invasive protrusions (Fig. 4B, arrows), suggesting that heterodimerization may promote three-dimensional invasive behavior. However, the phenotype was not readily apparent (Fig. 4B). In the past, we have reported that cooperation between ErbB2 signaling and TGF-β to promote invasion of three-dimensional acini is observed better when the three-dimensional acini were formed in Matrigel-collagen mix (25) and not on pure Matrigel. This is consistent with several recent reports that suggest that matrix composition can regulate malignant behavior of transformed cells (26, 27). Thus, we determined whether altered matrix composition can be used to create a permissive environment to monitor invasive behavior in response to ErbB1-ErbB2 heterodimer activation. Addition of AP1510 induced a modest effect on generation of invasive protrusions (Fig. 4C, AP1510) in cells plated on Matrigel-collagen mixture, whereas stimulation with rapalog induced dramatic effect on invasion of acinar structures and formation of interstructure bridges (Fig. 4C, rapalog). In addition, the structures were immunostained with anti–Laminin V antibodies to monitor changes in integrity of basement membrane surrounding three-dimensional structures. Activation of ErbB2 homodimers did not induce disruption of the Laminin V layer around the abnormal structures (Fig. 4D, AP1510), whereas activation of ErbB2-ErbB1 heterodimers induced a significant disruption of Laminin V basement membrane layer around the abnormal structures (Fig. 4D, rapalog). These observations show that ErbB1-ErbB2 heterodimers, but not ErbB2 homodimers, possess the ability to promote invasion of human mammary epithelial cells grown on three-dimensional matrix.

Heterodimer-induced invasion is sensitive to ErbB1 kinase activity and inhibition of signaling pathways. To gain insight into the possible role for ErbB1 in ErbB1-ErbB2–induced invasion, we induced heterodimerization in the presence of ErbB1-specific kinase inhibitor, ZD1839. We first determined whether the concentration of inhibitor used shows selectivity toward ErbB1 and is not nonspecifically inhibiting ErbB2 kinase activity also. Presence of ZD1839 did not significantly affect AP1510 or rapamycin–induced phosphorylation of ErbB2 (Fig. 5). However, presence of 1.0 μmol/L ZD1839 significantly inhibited rapamycin–induced phosphorylation of ErbB1 (Fig. 5). It should be noted that use of high concentrations (10.0 μmol/L) of ZD1839 significantly affected phosphorylation of both ErbB1 and ErbB2 (data not shown) and is consistent with what we have previously reported using Rat1 fibroblasts expressing chimeric ErbB receptors (28). Thus, we determined that ZD1839, at concentrations 1.0 μmol/L or lower, shows selectivity toward inhibition of ErbB1 receptor phosphorylation.

To determine the contribution of ErbB1 in an ErbB1-ErbB2 heterodimer–induced invasion, we turned to a more traditional and quantitative method of investigating cell invasion—ability of cells to invade through a layer of Matrigel-coated 8.0 μm pore polyethylene terephthalate membranes. Activation of ErbB1-ErbB2 heterodimers promoted a 4-fold increase in the number of cells invading through the Matrigel, whereas the presence of 0.5 to

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**Figure 4.** Heterodimer-induced proliferation and invasion. A, 10A.ErbB2/B1 cells were starved overnight and stimulated with rapalog or AP1510 for 18 to 20 hours before fixing and analyzing by flow cytometry. The percentage of cells in S phase was plotted. Average of three experiments done in duplicates. B, 10A.ErbB2/B1 cells were plated on Matrigel and allowed to undergo morphogenesis in the absence of synthetic ligands. Three-dimensional acinar structures were stimulated with either AP1510 or rapalog for 48 hours and phase images were recorded. Abnormal structures were identified as those that have three or more acinar structures abnormally organized (e.g., see inset in AP1510 image). Percentages of multiancinar structures were determined by scoring >200 acini for each condition. C, 10A.ErbB2/B1 cells were plated on a matrix that was generated by mixing Matrigel and collagen I in a ratio of 1:1. The structures were stimulated on day 4 and images were recorded on day 6. D, the structures were fixed and immunostained with anti–Laminin V (red) antibodies and counterstained with 4',6-diamidino-2-phenylindole to visualize the nuclei.

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**Figure 5.** Inhibition of ErbB1 in an ErbB1-ErbB2 heterodimer. 10A.ErbB2/B1 cells were stimulated with AP1510 or rapalog in the presence of increasing concentrations of ZD1839. The lysates were immunoprecipitated with anti-Glu-Glu or anti-HA antibodies and immunoblotted for antiphosphorytosine (pY) antibodies. The blots were reprobed with anti-Glu-Glu or anti-HA antibodies to serve as control.
1.0 μmol/L ZD1839 significantly inhibited the ability of ErbB1-ErbB2 heterodimer to promote invasion (Fig. 6). Because ErbB2 phosphorylation in a heterodimer was not significantly affected by 1.0 μmol/L ZD1839 (Fig. 5), these observations further show that activation of ErbB2 was not sufficient to promote invasion and suggest that ErbB1 kinase activity is required for ErbB1-ErbB2 heterodimer–induced invasion. The clinical implication of this observation is discussed below.

Several signaling pathways have been implicated in migration and invasion of mammary epithelial cells. Both the MAPK and the PI3K pathways are known to play important roles in ErbB-mediated migration/invasion of breast epithelial cells (29). To determine if controlled activation of ErbB1-ErbB2 heterodimer–induced invasion is sensitive to signaling pathways that have been shown to regulate invasion, we activated heterodimerization in the presence U0126 and LY29004, inhibitors for MAP/ERK kinase (MEK) and PI3K pathways, respectively, and observed a dose-dependent inhibition of heterodimer-induced invasion (Fig. 6). It should be noted that the concentrations of the chemical inhibitors used are low enough to specifically monitor contribution of the signaling pathway in question. These results also suggest that although Ras/MAPK and PI3K pathways are critical for regulating heterodimer-induced invasion, they may not be sufficient because ErbB2 fails to promote invasion albeit being able to activate these pathways (Fig. 3D).

However, PLCγ1 was specifically recruited to ErbB1-ErbB2 heterodimers and not to ErbB2 homodimers. We investigated whether ErbB1-ErbB2 heterodimer–induced invasion was sensitive to inhibition of PLCγ1 signaling. A PLCγ1-specific inhibitor, U73122, significantly inhibited invasion of MCF-10A structures, whereas an inactive analogue, U73343, did not affect ErbB1-ErbB2–induced invasion, suggesting that PLCγ1 plays a critical role in ErbB1-ErbB2 heterodimer–induced invasion. Because ErbB2 homodimers did not associate with PLCγ1, it is possible that PLCγ1 plays a unique role in the context of ErbB1-ErbB2 heterodimers to promote invasion of mammary epithelial cells.

### Discussion

We have developed a novel ErbB receptor dimerization system to specifically activate receptor heterodimers. Using such a system, we show that in addition to the ability of ErbB2 to cooperate with ErbB1 to promote proliferation, ErbB2 gains the ability to promote invasive behavior in human mammary epithelial cells when it heterodimerizes with the ErbB1 receptor.

We have previously shown that MCF-10A cells grown on three-dimensional matrix undergo a program of proliferation and apoptosis to form proliferation-arrested, organized epithelial structures that share several properties with breast epithelial acini (6). These in vitro three-dimensional acini-like structures provide a developmental context and serve as an important tool to study the biological effects of oncogenic signals. Most oncogenic signals that promote proliferative signals have the ability disrupt acini organization with oncogene-specific features (23). For instance, whereas activation of ErbB2 induces formation of abnormal noninvasive structures consisting of individual units, overexpression of CSF-1R induces large filled structures, overexpression of the HGF-MET induces development of abnormal, invasive structures, and overexpression of cyclin D1 and Akt results in formation of large acini (23). Our results suggest that activation of ErbB1-ErbB2 heterodimers was 2-fold more effective in its ability to induce formation of abnormal structures. Because formation of abnormal structures is a hallmark of aberrant ErbB2 signaling, we conclude that heterodimerization does not alter the oncogenic characteristic of ErbB2 but potentiates its oncogenic signaling.

Apart from a quantitative regulation of proliferation, it is not well established whether heterodimers differentially regulate other biological process. Our results using a controlled dimerization system show that ErbB1-ErbB2 heterodimers qualitatively differ from ErbB2 homodimers by promoting invasion of breast epithelial cells. This is unlikely to be a random effect of forced dimerization because neither homodimerization of ErbB2 nor addition of rapalog to parental 10A.ErbB2 cells promote invasive behavior. More importantly, inhibition of MEK and PI3K inhibited ErbB1-ErbB2 heterodimer-induced invasion, which is consistent with previous studies demonstrating important role for the PI3K and Ras/MAPK pathways in promoting cell migration (16, 30).

Despite the critical role played by PI3K and Ras/MAPK pathways in ErbB1-ErbB2 heterodimer–induced invasion, it is likely that they are not sufficient for the ability of ErbB receptors to promote invasion, because ErbB2 homodimers fail to induce invasion but retain the ability to activate Ras/MAPK and PI3K signaling pathways (Fig. 3D). Although heterodimers were more potent in their ability to activate PI3K signaling than ErbB2 homodimers (Fig. 3D), we are intrigued by the ability of ErbB1-ErbB2 heterodimers to uniquely associate with PLCγ1. Several reports have

![Figure 6. Heterodimer-induced invasion is sensitive to inhibition ErbB1 and selected signaling pathways.](image-url)
documented a role for PLCγ1 in cell migration (22). Activation of PLCγ1 is known to modulate intracellular calcium levels and activation of protein kinase C, both regulate cell shape and cell motility (22). PLCγ1 is thought to play a role in ErbB-induced migration of breast cancer cells in culture (31, 32) and is also known to be overexpressed in breast cancer (33). Our observations suggest a unique role for PLCγ1 in ErbB1-ErbB2 heterodimer-induced invasion of breast epithelial cells raising the possibility that inhibition of PLCγ1 can have specificity for ErbB-overexpressing breast cancers.

Previous reports have suggested that overexpression of ErbB2, by itself, can promote invasive behavior in cultured breast epithelial cells (30) and in transgenic mouse models (4). Although these studies suggest that ErbB2 homodimer may be sufficient to promote invasive behavior, it is unlikely to be the case. As active ErbB2 signaling results in autocrine production EGF family ligands, such as amphiregulin, in epithelial cells, including MCF-10A (34), it will promote activation of ErbB1-ErbB2 heterodimers involving ErbB1 receptors that is expressed in all epithelial cells. Thus, it is not possible to understand the relative contribution of homodimers and heterodimers in promoting invasion. Our studies compared the effects of homodimers and heterodimers in the absence of any other receptor dimers to conclude that the ability of cells to invade is controlled by signals generated by ErbB1-ErbB2 heterodimers, thus squarely placing invasive behavior downstream of ErbB1-ErbB2 heterodimer signaling. Our conclusions further imply that up-regulation of ErbB1 expression can be a critical step in progression of ErbB2-positive cancers and is consistent with clinical studies that show that coexpression of ErbB1 and ErbB2 correlates with poor clinical outcome (11).

Relationship between ErbB1 and ErbB2 coexpression and poor clinical prognosis raises the possibility that inhibiting ErbB1 kinase activity can be beneficial for treating ErbB2-positive tumors. In fact, drugs targeting multiple ErbB receptors are more effective than targeting one ErbB receptor (35, 36). Lapatinib, a reversible inhibitor of ErbB1 and ErbB2, can both synergize with trastuzumab (Herceptin), a monoclonal antibody that inhibits ErbB2 activity to induce apoptosis in ErbB2-overexpressing breast cancer cells (37), and treat trastuzumab-refractory cancers (38). In addition, pertuzumab, an antibody that blocks heterodimerization of ErbB2, is effective in model systems and in the clinic (39, 40). Because our studies suggest that ErbB2 gains the ability to induce invasion when heterodimerized with ErbB1, we asked whether the cooperative effect of targeting multiple ErbB receptors relates to the ability of heterodimers to promote invasion. Our results show ErbB1 kinase activity is required for heterodimer-induced invasion of mammary epithelial cells and further suggests that targeting ErbB receptor heterodimerization can be more effective than targeting a single ErbB receptor. Moreover, our studies also show that characterization of novel signaling pathways by ErbB1-ErbB2 heterodimer receptor heterodimerization, such as PLCγ1 activation, can identify molecules that serve as targets for more effective treatments.

Thus, we have characterized a novel controlled dimerization system to activate ErbB1-ErbB2 receptor heterodimerization and identify a heterodimer-specific biological effects providing important insights into the mechanisms by which ErbB2 overexpressing ductal carcinoma in situ can gain invasive properties. In addition, we have established a system that can be extended to investigate biological and biochemical specificities of other receptor heterodimers within the ErbB family or other receptor tyrosine kinases, which can lead to identification of novel biomarkers and drug targets.

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Lixing Zhan, Bin Xiang and Senthil K. Muthuswamy