Constitutively Active ErbB4 and ErbB2 Mutants Exhibit Distinct Biological Activities¹

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Abstract
ErbB4 is a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinases, which includes EGFR/ErbB1, ErbB2/HER2/Neu, and ErbB3/HER3. These receptors play important roles both in normal development and in neoplasia. For example, deregulated signaling by ErbB1 and ErbB2 is observed in many human malignancies. In contrast, the roles that ErbB4 plays in tumorigenesis and normal biological processes have not been clearly defined. To identify the biological responses that are coupled to ErbB4, we have constructed three constitutively active ErbB4 mutants. Unlike a constitutively active ErbB2 mutant, the ErbB4 mutants are not coupled to increased cell proliferation, loss of contact inhibition, or anchorage independence in a rodent fibroblast cell line. This suggests that ErbB2 and ErbB4 may play distinct roles in tumorigenesis in vivo.

Introduction
ErbB4 (HER4/p180EGF-BH4) is a member of the EGFR² (EGFR/ErbB) family of receptor tyrosine kinases. These receptors play important roles in the embryonic development of heart, lung, and nervous tissues (1–4), and they have been implicated in the progression of metastatic disease. For example, EGFR/ErbB1 is overexpressed, amplified, or mutated in a number of human malignancies including breast, ovary, prostate, and lung cancers (5–7). ErbB2 overexpression correlates with tumor aggressiveness and poor prognosis in node-positive breast cancer patients (reviewed in Ref. 8). Finally, ErbB3 overexpression is observed in a subset of human mammary and gastric cancers (9, 10).

Some reports indicate that increased ErbB4 expression or signaling is associated with tumorigenesis. ErbB4 overexpression has been observed in a variety of cancers, including tumors of the thyroid, breast, and gastrointestinal tract (11–14). However, the prognostic significance of ErbB4 expression in tumors may also depend on which Erbb family members are coexpressed with ErbB4. In the case of childhood medulloblastoma (one of the most common solid tumors of childhood), patients with tumors overexpressing both ErbB2 and ErbB4 have a significantly worse prognosis than patients with tumors that overexpress either receptor alone (15).

Other reports indicate that increased ErbB4 expression or signaling correlates with tumor cell differentiation and reduced aggressiveness. ErbB4 overexpression in breast tumors is associated with progesterone receptor and estrogen receptor expression and a more favorable prognosis (16–17). In contrast, ErbB2 overexpression varies inversely with progesterone receptor and estrogen receptor levels and indicates tumors that are more likely to be metastatic and fatal (18). In one survey of common solid human cancers, the loss of ErbB4 expression is seen in a significant percentage of breast, prostate, and head and neck malignancies (19). These findings raise the intriguing possibility that ErbB4 is unique to the ErbB family of receptors in that ErbB4 expression and signaling may couple to reduced tumorigenesis or tumor cell proliferation. However, in the face of the conflicting evidence we have summarized here, it remains unclear what general or specific roles ErbB4 plays in differentiation, tumor suppression, or proliferation.

Efforts to elucidate ErbB4 function have been hampered by many factors. There are no known agonists or antagonists specific to the ErbB4 receptor. All of the peptide hormones of the EGF family that are capable of binding ErbB4 also bind at least one other ErbB family member. For example, epiregulin and betacellulin bind and activate both ErbB1 and ErbB4 (20, 21). Furthermore, ligands that do not bind an ErbB family receptor can still activate signaling by that receptor in trans through ligand-induced receptor heterodimerization (reviewed in Refs. 22, 23). For example, EGF stimulates ErbB2 tyrosine phosphorylation when ErbB2 is coexpressed with ErbB1, whereas EGF will not stimulate ErbB2 tyrosine phosphorylation in the absence of ErbB1 (24). Consequently, ligands that bind and directly activate ErbB4 (neuregulin, betacellulin, and epiregulin) also stimulate ErbB1, ErbB2, and ErbB3 signaling (Refs. 20, 21, 25, 26; reviewed in Refs. 22, 23). Therefore, in most contexts it is virtually impossible to use an EGF family hormone to study the functional consequences of ErbB4 signaling.

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³ The abbreviations used are: EGFR, epidermal growth factor receptor; cfu, colony-forming unit(s); FR3T3, Fischer rat 3T3; LMP, low melting point; LTR, long terminal repeat.
Constitutively Active ErbB4 Mutants

To study ErbB4 function, we have opted to generate ErbB4 mutants that contain a cysteine substitution in the extracellular domain. This is predicted to result in constitutively dimerized and constitutively active ErbB4 mutants. Introducing cysteine residues to form covalently linked, dimeric, constitutively active receptor tyrosine kinases is not novel. This strategy has been used to generate dimeric, constitutively active mutants of EGFR/ErbB1 and ErbB2 (27, 28). Cysteine substitutions also lead to constitutively active mutants of the fibroblast growth factor receptors 2 and 3 (29, 30).

Here we report the generation and characterization of three constitutively active ErbB4 mutants. These mutants were generated through the introduction of a cysteine residue in the extracellular region of ErbB4. These mutants exhibit increased ligand-independent ErbB4 tyrosine phosphorylation, dimerization, and kinase activity. However, these constitutively active ErbB4 mutants do not induce increased proliferation, loss of contact inhibition, or anchorage-independent growth in FR3T3 fibroblasts. In contrast, a constitutively active ErbB2 mutant does induce increased proliferation, loss of contact inhibition, and anchorage-independent growth in FR3T3 fibroblasts. These results suggest that ErbB4 and ErbB2 couple to different signaling pathways and biological responses. These results also suggest that ErbB4 and ErbB2 may play distinct roles in tumorigenesis in vivo.

Results

ErbB4 Mutants Are Constitutively Tyrosine Phosphorylated. We substituted a single cysteine for amino acids Pro-645, Gin-646, His-647, Ala-648, and Arg-649 in the juxtamembrane region of the ErbB4 extracellular domain. These ErbB4 mutants (P645C, Q646C, H647C, A648C, and R649C) were generated in the context of the pLXSN-ErbB4 recombinant retroviral expression vector (26). Because these cysteine substitutions might cause inappropriate protein folding and decreased protein stability, we assayed the ErbB4 mutants for stable expression. We transfected the recombinant retroviral vectors containing the ErbB4 mutant constructs into the V2 ecotropic retrovirus packaging cell line, selected for stable transformants, and generated pooled cell lines. We harvested low-titer ecotropic retrovirus stocks from these cell lines, and we analyzed the expression and tyrosine phosphorylation of the ErbB4 mutants in these cell lines. Three ErbB4 mutants (Q646C, H647C, and A648C) exhibit abundant expression and ligand-independent tyrosine phosphorylation (data not shown). However, the R649C ErbB4 mutant is not efficiently expressed, and the P645C mutant does not display ligand-independent tyrosine phosphorylation (data not shown).

Previous studies indicate that transfection and subsequent overexpression of ErbB family receptors lead to ligand-independent receptor tyrosine phosphorylation (31–33). Consequently, we were concerned that the ligand-independent phosphorylation of the Q646C, H647C, and A648C ErbB4 mutants in the transfected V2 cells was a consequence of overexpression. Therefore, we infected the PA317 amphotropic retrovirus packaging cell line with the ErbB4 mutant recombinant ecotropic retroviruses at low multiplicities of infection (<0.1), selected for infected cells, and generated pooled cell lines. Because these cell lines were generated by infection at low multiplicities of infection, it is likely that each cell contains only one or two copies of the ErbB4 expression construct. This reduces the likelihood of ErbB4 overexpression in these cell lines.

We analyzed ErbB4 expression and tyrosine phosphorylation in the PA317 cell lines by anti-ErbB4 immunoprecipitation and either anti-ErbB4 (Fig. 1, right panel) or anti-phosphotyrosine (Fig. 1, left panel) immunoblotting. As expected, cells infected with the LXSN vector control retrovirus do not exhibit ErbB4 expression (Fig. 1, right panel) or tyrosine phosphorylation (Fig. 1, left panel). Cells infected with the wild-type or mutant ErbB4 retroviruses exhibit ErbB4 expression (Fig. 1, right panel). However, cells infected with the mutant ErbB4 retroviruses exhibit abundant ErbB4 tyrosine phosphorylation, whereas cells infected with the wild-type ErbB4 retrovirus exhibit minimal ErbB4 tyrosine phosphorylation (Fig. 1, left panel).

Quantification of the chemilumigrams shown in Fig. 1 suggests that the expression levels of the three ErbB4 mutants is less than three times greater than the amount of wild-type ErbB4 expression (Table 1). In contrast, the amounts of tyrosine phosphorylation of the three ErbB4 mutants appear to be much greater than the amount of wild-type ErbB4 tyrosine phosphorylation. Moreover, the ratios of ErbB4 tyrosine phosphorylation to ErbB4 expression for the three ErbB4 mutants appear to be at least four times greater than the ratio for wild-type ErbB4. These data suggest that the three ErbB4 mutants exhibit greater amounts of tyrosine phosphorylation on a per-molecule basis than does wild-type ErbB4. Consequently, these data indicate that the Q646C, H647C, and A648C ErbB4 mutants are constitutively active for signaling.
ErbB4 Mutants Have Increased in Vitro Kinase Activity.

Next, we assessed whether the increased tyrosine phosphorylation of the three ErbB4 mutants correlates with increased kinase activity. Equal amounts of the same lysates used for the experiments described in Fig. 1 were immunoprecipitated with an anti-ErbB4 polyclonal antibody. Kinase reactions were performed on the immunoprecipitates in the presence of \([\gamma-32\text{P}]\text{ATP}\). The reaction products were resolved by SDS-PAGE on a 7.5% acrylamide gel. The gel was dried, and the reaction products were visualized by autoradiography.

In Fig. 2, we show that PA317 cells infected with the LXSN vector control retrovirus lack detectable ErbB4 kinase activity. Moreover, PA317 cells that express the three constitutively active ErbB4 mutants exhibit greater ErbB4 tyrosine kinase activity than cells that express wild-type ErbB4. Quantification of the bands on the autoradiogram indicates that the Q646C and H647C ErbB4 mutants exhibit approximately five times more kinase activity than does wild-type ErbB4, whereas the A648C ErbB4 mutant exhibits approximately nine times more kinase activity than does wild-type ErbB4. Given that the expression of the ErbB4 mutants (in these same lysates) is somewhat greater than the expression of wild-type ErbB4 (Fig. 1 and Table 1), it appears that the intrinsic kinase activity of the three ErbB4 mutants is three to four times greater than the intrinsic kinase activity of wild-type ErbB4.

Constitutively Active ErbB4 Mutants Do Not Induce a Loss of Contact Inhibition. Once we determined that the Q646C, H647C, and A648C ErbB4 mutants are constitutively active for signaling, we performed experiments using these mutants to identify the biological events coupled to ErbB4 signaling. A common assay for genes that encode growth control or signaling proteins involves introducing the gene into an established rodent fibroblast cell line and assaying for foci of piled-up cells. These foci indicate a loss of contact inhibition, a common attribute of malignant cells. Thus, this gene transfer assay is commonly used to identify genes that encode proteins that are coupled to malignant growth transformation.

Conflicting results have been obtained from assays for growth transformation by ErbB4. Transfection and consequent overexpression of ErbB4 induces foci (loss of contact inhibition) in NIH 3T3 clone 7 cells in the absence of ligand. Moreover, in these cells focus formation was stimulated by the ErbB4 ligand neuregulin 2β. In contrast, NIH 3T3 clone 7d cells (which lack EGFR expression) transfected with wild-type ErbB4 did not form foci in the presence or absence of neuregulin 1β; however, ErbB4 cotransfected with EGFR/ErbB1 or ErbB2 does induce foci in these cells (32, 33). One possible explanation is that ErbB4 lacks intrinsic transforming activity but does permit EGFR/ErbB1 or ErbB2 signaling and coupling to growth transformation in the presence of an ErbB4 ligand.

To test whether ErbB4 signaling is sufficient to transform the growth of cultured rodent fibroblasts, FR3T3 fibroblasts were infected with 200 cfu of the ErbB4 mutant recombinant ecotropic retrovirus stocks and assayed for focus formation. Cells infected with 200 cfu of the LXSN vector control recombinant ecotropic retrovirus and with 200 cfu of the wild-type ErbB4 recombinant ecotropic retrovirus served as negative controls. Cells infected with 200 cfu of the constitutively active (V664E transmembrane domain) mutant ErbB2* retrovirus served as a positive control.

FR3T3 cells infected with the ErbB2* retrovirus had formed foci within 9 days after infection, whereas cells infected with the vector control retrovirus had not (Fig. 3). Furthermore, cells infected with the wild-type or mutant ErbB4 retroviruses had not formed foci within 9 days after infection. Within 18 days after infection, the foci arising from FR3T3 cells infected with the ErbB2* retrovirus had completely covered the surface of the tissue culture plate and had begun to detach from the surface of the plate (data not shown). Within 18 days after infection, FR3T3 cells infected with the mutant ErbB4 retroviruses had formed relatively high-density clumps (data not shown). These high-density clumps did not exhibit the overlapping cell processes characteristic of foci (data not shown). The cells comprising these clumps were cloned and expanded into cell lines, as were cells from less dense regions of the cell monolayers. The cells from the clumps are morphologically indistinguishable from cells derived from the

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Constitutively Active ErbB4 Mutants Do Not Induce Anchorage-independent Growth. Next, we assayed FR3T3 cells that express the constitutively active ErbB4 mutants for growth while suspended in semisolid medium. Because anchorage-independent growth is another characteristic attribute of tumor cells in vivo, this assay is another way to determine whether ErbB4 signaling is coupled to malignant growth transformation.

FR3T3 cells were infected with the ErbB4 mutant recombinant ecotropic retroviruses at a low multiplicity of infection, and infected cells were selected using G418. Drug-resistant colonies of cells were pooled and expanded into cell lines. Control cell lines were generated through infection of FR3T3 cells with the wild-type ErbB4 retrovirus, the constitutively active ErbB2 retrovirus, and with the LXSN vector control retrovirus. These cell lines were seeded at a density of $2 \times 10^5$ cells/ml in 60-mm dishes in semisolid medium containing 0.3% LMP-agarose. Fresh medium containing LMP-agarose was added every 3 days. Photographs were taken of representative fields after 10 days.

FR3T3 cells that express the constitutively active ErbB2* mutant exhibit anchorage-independent growth (Fig. 4). In contrast, cells that were infected with the LXSN recombinant retroviral vector control and cells that express wild-type ErbB4 or the ErbB4 mutants do not exhibit anchorage-independent growth. The results of this assay are consistent with the results of the focus formation assay; both assays indicate that ErbB4 signaling is distinct from ErbB2 signaling in that ErbB4 signaling is not coupled to malignant growth transformation in FR3T3 fibroblasts.

**Constitutively Active ErbB4 Mutants Do Not Increase the Growth Rate or Saturation Density.** Another characteristic of malignant transformed fibroblasts is that their growth rates and saturation densities are higher than those of their nontransformed counterparts. Indeed, constitutive ErbB2 signaling is coupled to increased growth rates (reviewed in Ref. 8). Thus, we assessed whether the constitutively active ErbB4 mutants affected the growth rate or saturation density of FR3T3 fibroblasts. The FR3T3 cell lines described earlier were seeded in 60-mm dishes at a density of $2 \times 10^4$ cells/dish (700 cells/cm²). Cells were incubated for 10 days to permit proliferation. During this period, cells were counted every 24 h.

The growth rate of the cells that express ErbB2* is slightly greater than the growth rates of the other cell lines (Fig. 5). Note that the growth rates of the cells that express the constitutively active ErbB4 mutants are indistinguishable from the growth rates of cell lines that express wild-type ErbB4 or the vector control. The growth curves in Fig. 5 were used to determine the saturation densities for the six cell lines (Table 2). Note that the saturation density of the cell line that expresses ErbB2* is higher than the saturation densities of the other cell lines. Moreover, the saturation densities of the cell lines that express the ErbB4 mutants are not markedly higher than the saturation densities of the vector control cell line or the cell line that expresses wild-type ErbB4. Once again, these data suggest that constitutive ErbB4 signaling is not coupled to malignant growth transformation in fibroblasts. Thus, the signaling pathways and biological responses that are coupled to ErbB4 are distinct from those that are coupled to ErbB2.

**The Constitutively Active ErbB4 Mutants Are Expressed and Are Constitutively Tyrosine Phosphorylated in FR3T3 Cells.** We were concerned that the apparent failure of the constitutively active ErbB4 mutants to transform the growth of FR3T3 fibroblasts might be attributable to the absence of ErbB4 expression or constitutive tyrosine phosphorylation in these cells. In parallel with the infections described in Fig. 3, we infected FR3T3 cells with 200 cfu of the constitutively active mutant ErbB4 recombinant retroviruses...
and selected for stable infection using G418. As controls, we also infected FR3T3 cells with 200 cfu of the vector control retrovirus, 200 cfu of the ErbB2* retrovirus, and with 200 cfu of the wild-type ErbB4 retrovirus. Drug-resistant colonies were pooled and expanded into stable cell lines. The cell lines were starved of serum in the presence of 500 μM Na3VO4 (34) to decrease the background level of tyrosine phosphorylation and to increase the phosphorylation of the

Fig. 4. Constitutively active ErbB4 receptors do not induce growth in semisolid medium. FR3T3 cells that stably express the LXSN vector control, the constitutively active ErbB2 mutant (ErbB2*), wild-type ErbB4, or the constitutively active ErbB4 mutants (Q646C, H647C, and A648C) were seeded in semisolid medium at a density of 2 × 10⁴ cells/ml in 60-mm dishes. The cells were incubated for 10 days, after which images were recorded by photomicroscopy. Images shown are representative of those obtained in three independent experiments.

Fig. 5. Constitutively active ErbB4 mutants do not increase the growth rate of FR3T3 fibroblasts. FR3T3 cells that express the LXSN vector control, the constitutively active ErbB2* mutant, wild-type ErbB4, or the constitutively active ErbB4 mutants (Q646C, H647C, and A648C) were plated at a density of 2 × 10⁵ cells in 60-mm dishes (100 cells/cm²) and were incubated for 1–10 days. Cells were counted daily to assess growth rates and saturation densities. The means for three independent experiments; bars, SE.
constitutively active ErbB4 mutants. We prepared lysates and analyzed ErbB4 expression and tyrosine phosphorylation by precipitation with an anti-ErbB4 antibody and sequential anti-phosphotyrosine and anti-ErbB4 immunoblotting.

In Fig. 6, lower panel, we show that ErbB4 expression is detectable in the FR3T3 cell lines infected with the wild-type ErbB4 retrovirus or the constitutively active ErbB4 mutant retroviruses. However, ErbB4 tyrosine phosphorylation is observed only in the FR3T3 cell lines infected with the constitutively active ErbB4 mutant retroviruses (Fig. 6, upper panel). The amount of phosphorylation exhibited by the ErbB4 mutants is less than the amount of phosphorylation exhibited by the constitutively active ErbB2 mutant. Furthermore, the expression of wild-type ErbB4 appears to be less than the expression of the ErbB4 mutants. Nonetheless, these data suggest that the apparent failure of the constitutively active ErbB4 mutants to transform the growth of FR3T3 fibroblasts is not attributable to an absence of expression and tyrosine phosphorylation of these mutants in these cells.

Discussion
In this report, we describe the construction and initial characterization of three constitutively active ErbB4 mutants. These mutants display increased dimerization (data not shown) and ligand-independent tyrosine phosphorylation and kinase activity. In these respects, the ErbB4 mutants resemble constitutively active mutants of ErbB2 or EGFR. However, unlike constitutively active ErbB2 mutants, these mutants are not coupled to malignant growth transformation in FR3T3 fibroblasts; they do not induce foci, anchorage-independent growth, or increases in the growth rate or saturation density. These data suggest that ErbB2 and ErbB4 play distinct roles in tumorigenesis in vivo. This conclusion is supported by the observation that NIH3T3 clone 7d cells do not form foci after ErbB4 transfection and treatment with the ErbB4 ligand neuregulin but do form foci after ErbB2 and ErbB4 cotransfection and neuregulin treatment (32, 33).

Of course, another potential explanation is that the amounts of tyrosine phosphorylation displayed by the three constitutively active ErbB4 mutants are insufficient to couple to malignant growth transformation in fibroblasts. This is consistent with the observation that the three constitutively active ErbB4 mutants are less phosphorylated than the constitutively active ErbB2 mutant (Fig. 6). However, anti-phosphotyrosine immunoblotting is not a sensitive method for assessing ErbB family receptor signaling and coupling to biological responses. Indeed, the neuregulin concentration required for maximal ErbB4 tyrosine phosphorylation is ~10-fold greater than the neuregulin concentration sufficient for maximal ErbB family receptor coupling to biological responses. Furthermore, the neuregulin concentration sufficient for maximal ErbB family receptor coupling to biological responses stimulates, at most, only modest amounts of ErbB4 tyrosine phosphorylation (26). Thus, it is not likely that the failure of the constitutively active ErbB4 mutants to couple to malignant growth transformation in fibroblasts is attributable to insufficient ErbB4 tyrosine phosphorylation. Clearly, additional work is necessary to define the roles that ErbB4 plays in tumorigenesis and in regulating cellular functions in vivo. However, important clues have emerged to guide these future studies. In a significant percentage of breast tumor samples, ErbB4 expression correlates with estrogen receptor expression, which indicates a favorable prognosis (16–17). Furthermore, ErbB4 expression is fre-
quently lost in tumors of the breast and prostate (19). Finally, ligands for ErbB4 can induce terminal differentiation and growth arrest of some mammary tumor cell lines (35–37). These data indicate that ErbB4 signaling may be coupled to differentiation, growth arrest, and tumor suppression. The ErbB4 mutants described in this study will enable us to evaluate this hypothesis. Indeed, preliminary data from our laboratory indicate that the Q646C ErbB4 mutant causes reduced colony formation in plastic dishes by a number of cultured human breast and prostate tumor cell lines.

We will also perform additional studies to characterize the biochemistry of signaling by the three ErbB4 mutants. Whereas these mutants exhibit greater ligand-independent tyrosine phosphorylation and autokinase activity than the wild-type receptor, it is unclear whether this is attributable to increased intrinsic kinase activity or attributable to increased availability of the substrate. Additional experiments are warranted to distinguish between these two possibilities.

Another area of future study will focus on identifying the mechanisms by which ErbB4 is coupled to biological responses. Initial studies will identify the sites of ErbB4 tyrosine phosphorylation for these mutants. If our preliminary studies indicating that the Q646C ErbB4 mutant is coupled to prostate and mammary tumor cell growth arrest hold true, then we will use genetic strategies to identify the sites of ErbB4 tyrosine phosphorylation that are sufficient and necessary to couple the Q646C ErbB4 mutant to this biological response. A similar strategy has been used to identify the sites of ErbB2 and platelet-derived growth factor receptor tyrosine phosphorylation that are critical for coupling these receptors to biological responses (38, 39).

Once we have identified the site(s) of tyrosine phosphorylation that is sufficient for coupling to biological responses, we will identify signaling proteins that bind this phosphorylation site and couple it to biological responses. Using this strategy, we will begin to characterize the ErbB4 signaling pathway. Our prediction is that the three constitutively active ErbB4 mutants are phosphorylated on different tyrosine residues and that these mutants differentially couple to biological responses. We have shown previously that different ErbB4 ligands cause phosphorylation on different sites on ErbB4 and differential coupling to biological responses (40). Moreover, one cysteine substitution mutation in the rat ErbB2 extracellular domain (V656C) results in low amounts of constitutive receptor tyrosine phosphorylation and efficient coupling to malignant growth transformation in rodent fibroblasts. In contrast, another rat ErbB2 extracellular domain cysteine substitution mutant (T657C) exhibits very high levels of constitutive receptor tyrosine phosphorylation but a relatively low amount of coupling to malignant growth transformation in rodent fibroblasts (28).

We were somewhat surprised to discover that the three constitutively active ErbB4 mutants failed to couple to malignant growth transformation in a rodent fibroblast cell line. Nonetheless, these mutants will enable us to assess ErbB4 function in a wide variety of cell, tissue, and organ ischemic contexts. Given that ErbB4 appears to regulate diverse functions in a number of distinct contexts, much work remains to complete this story.

Materials and Methods

Cell Lines, Cell Culture, and Antibodies. The Y2, PA317, C127, and FR3T3 cell lines were generous gifts from Daniel DiMaio (Yale University New Haven, CT). All cell lines were propagated in DMEM supplemented with 10% FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin (Mediatech), and 0.25 μg/ml Fungizone (Amphotericin B; Life Technologies, Inc.). Recombinant cell lines generated in the course of the experiments described in this report were propagated in the medium described above supplemented with 200 μg/ml G418 (Mediatech).

The anti-ErbB4 mouse monoclonal (SC-8050), anti-ErbB4 rabbit polyclonal (SC-283), and anti-ErbB2 rabbit polyclonal (C-18) antibodies were purchased from Santa Cruz Biotechnology. Goat antimouse and goat antirabbit horseradish peroxidase-conjugated antibodies were purchased from Pierce. Enhanced chemiluminescence (ECL) Western blotting reagents, Redivue adenosine 5′-[γ-32P]triphosphate, and Protein-A Sepharose (CL-4B) were purchased from Amersham Pharmacia Biotech. The 4G10 anti-phosphotyrosine mouse monoclonal antibody was purchased from Upstate Biotechnology.

Plasmids. The recombinant retroviral vector pLXSN (41) was obtained from Daniel DiMaio (Yale University). This construct contains two recombinant LTRs derived from the Moloney murine leukemia virus and the Moloney murine sarcoma virus. These LTRs flank the Y packaging signal and the amingoglycoside 3′-phosphotransferase (Neo<sup>B</sup>) gene under the transcriptional control of the SV40 early promoter. The Neo<sup>B</sup> gene confers resistance to the amingoglycoside antibiotic G418 (geneticin; Life Technologies, Inc.).

The recombinant retroviral construct pLXSN-ErbB4 (26) was generated by subcloning the human ErbB4 cDNA into pLXSN. In this construct, the ErbB4 cDNA is under the transcriptional control of the upstream LTR. The recombinant retroviral construct pLXSN-ErbB2<sup>Q</sup> (42) was a gift of Lisa Petti (Albany Medical College, Albany, NY). It was generated by subcloning the cDNA encoding the constitutively active rat ErbB2 mutant (V664E transmembrane domain mutant, ErbB2<sup>Q</sup>) into pLXSN. In this construct, the ErbB2<sup>Q</sup> cDNA is under the transcriptional control of the upstream LTR.

ErbB4 Mutagenesis. The plasmid pLXSN-ErbB4 was used as the template for site-directed mutagenesis (QuikChange Site Directed Mutagenesis kit; Stratagene) to construct the putative constitutively active ErbB4 mutants. The mutants were constructed by introducing mutations that substitute a cysteine residue for proline 645, glutamine 646, histidine 647, alanine 648, or arginine 649 in the ErbB4 extracellular juxtamembrane domain. These mutants are denoted as follows: P645C, Q646C, H647C, A648C, and R649C. A new restriction enzyme site was also engineered in the transcriptional control of the upstream LTR.

The following primers were used for mutagenesis. “T” denotes the upper primer, whereas “B” denotes the lower primer. The novel cysteine codons and anticodons are indicated by bold type, the point mutations that create the novel cysteine residues are double underlined, and the novel restriction enzyme sites are singly underlined.
Constitutively Active ErbB4 Mutants

Production of Recombinant Retroviruses and Retroviral Infections. The ErbB4 mutant constructs were transfected using standard techniques (44, 45) into the \( \frac{2}{3} \) eco-tropic retrovirus packaging cell line (46) to generate cell lines that express the ErbB4 mutants and to package the amphotropic packaging cell line (46) to generate cell lines that express the ErbB4 mutants and to package the amphotropic retrovirus as described earlier and in reports published previously (47). FR3T3 and C127 cells were infected with recombinant eco-tropic retroviruses as described earlier and in reports published previously (47).FR3T3 and C127 cells were infected with recombinant eco-tropic retroviruses as described earlier and in reports published previously (47).

Focus Formation Assay for Loss of Contact Inhibition. FR3T3 and C127 cells were infected with recombinant eco-tropic retroviruses as described earlier and in reports published previously (47).FR3T3 and C127 cells were infected with recombinant eco-tropic retroviruses as described earlier and in reports published previously (47).

Assay for Anchorage Independence. FR3T3 cells were seeded at a density of \( 2 \times 10^5 \) cells in 60-mm dishes containing 2.5 ml of 0.3% LMP-agarose (Life Technologies, Inc.)
as described previously (50). Every 3 days, DMEM supplemented with 10% FBS and 0.3% LMP-agarose was added to each plate. The cells were incubated at 37°C for 10 days, and fields were photographed with an Olympus OM-10 camera attached to an Olympus CK-2 phase-contrast inverted microscope. The images were digitized by the photofinisher. These images were cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software. Images are representative of three independent experiments.

**Growth Rate/Saturation Density Assay.** Stable FR3T3 cell lines expressing the wild-type ErbB4 receptor, ErbB2*, or the ErbB4 mutants (Q646C, H647C, and A648C) were plated in 10 60-mm dishes at a density of 2 × 10⁴ cells/dish. Cells were incubated from 1 to 10 days at 37°C. Cells were counted (Coulter Counter 2M) each day for a total of 10 days. The mean and SE are representative of three independent experiments.

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**References**


