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Phosphorylation of ErbB4 on Tyrosine 1056 Is Critical for ErbB4 Coupling to Inhibition of Colony Formation by Human Mammary Cell Lines

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Abstract

In many studies, ErbB4 expression in breast tumor samples correlates with a favorable patient prognosis. Similarly, ErbB4 signaling is coupled to cellular differentiation and growth arrest in a variety of model systems. However, in some studies, ErbB4 expression in breast tumor samples correlates with poor outcome. Likewise, studies using some human mammary tumor cell lines suggest that ErbB4 is coupled to malignant phenotypes. Thus, the roles that ErbB4 plays in human breast cancer are still poorly defined. Here we demonstrate that a constitutively active ErbB4 mutant (ErbB4-Q646C) inhibits colony formation on plastic by two human mammary tumor cell lines (SKBR3 and MCF7) and by the MCF10A immortalized human mammary cell line, but does not inhibit colony formation by the MDA-MB-453 and T47D human mammary tumor cell lines. ErbB4 kinase activity is necessary for ErbB4 function and phosphorylation of ErbB4 Tyr1056 is necessary and appears to be sufficient for ErbB4 function. The inhibition of colony formation by MCF10A cells is accompanied by growth arrest but not cell death. These data suggest that ErbB4 behaves as a mammary tumor suppressor and that loss of ErbB4 coupling to growth arrest may be an important event in mammary tumorigenesis.

Keywords

ErbB4; Breast cancer; Signal transduction; Tumor suppressor; Receptor tyrosine kinase

INTRODUCTION

ErbB4 is a member of the ErbB family of receptor tyrosine kinases. This family includes the epidermal growth factor receptor (EGFR/HER1/ErbB1), ErbB2 (HER2/Neu), ErbB3 (HER3), and ErbB4 (HER4) (1–4). These receptors share 50% amino acid identity and many functional and structural features. Each possesses an extracellular ligand binding domain, a single-pass transmembrane domain, and a cytoplasmic tyrosine kinase domain (5). Agonists for these receptors are members of the epidermal growth factor (EGF) family of peptide hormones and include amphiregulin, transforming growth factor-α (TGF-α), betacellulin, heparin-binding EGF-like growth factor (HB-EGF), epiregulin, and members of the heregulin/neuregulin subfamily. Upon ligand binding, ErbB receptors form homo- or heterodimers that become

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cross-phosphorylated on tyrosine residues and couple to downstream signaling cascades (6–8).

EGFR and ErbB2 signaling couple to mammary cell proliferation and tumorigenesis. EGFR overexpression is an indicator for recurrent breast tumors and correlates with both a shorter period of disease-free survival and with a shorter period of overall survival. Likewise, in breast cancer patients ErbB2 overexpression correlates with increased tumor size, spread to the lymph nodes, and poor patient outcome (7,9).

ErbB4 is expressed in the normal mammary epithelium (10,11). ErbB4 expression varies in human breast tumor samples but is typically absent or markedly reduced compared to normal breast tissue samples in up to 89% of these samples (10,12–15). Moreover, in several studies of human breast tumor samples ErbB4 expression correlates with a favorable prognosis or with markers for a favorable prognosis (11–18). These data indicate that ErbB4 may couple to mammary cell growth inhibition and tumor suppression or that ErbB4 expression may be a marker for these events.

In some human breast tumor cell lines, ErbB4 signaling couples to cellular differentiation and growth arrest. Indeed, the neuregulin (heregulin) ErbB4 ligands are also known as Neu differentiation factors because they induce differentiation and growth arrest by human breast tumor cell lines (19,20). Ectopic expression of ErbB4 in a human breast tumor cell line that lacks endogenous ErbB4 results in neuregulin 1β stimulation of differentiation and growth arrest. Similarly, a dominant-negative ErbB4 mutant prevents neuregulin 1β from inhibiting the proliferation of a human breast tumor cell line that endogenously expresses ErbB4 (21). These data suggest that ErbB4 signaling is coupled to mammary cell differentiation, growth arrest, and tumor suppression.

In mice, ErbB4 expression in the mammary gland is highest during a period late in pregnancy that is characterized by terminal differentiation and growth arrest (22). Indeed, the ErbB4 agonist neuregulin 1β induces the differentiation of the mammary epithelium into lobuloalveoli that exhibit milk protein expression (23) and expression of a dominant-negative ErbB4 transgene in the mouse mammary gland disrupts normal lactation (24). Thus, these data suggest that ErbB4 is coupled to mammary cell differentiation and growth arrest.

Other studies indicate that ErbB4 may behave as an oncogene in mammary tissues. However, the interpretation of these data does not appear to be straightforward. Two clinical studies indicate that ErbB4 expression correlates with a poor patient prognosis (25,26). One factor that may affect the general applicability of these findings is that these studies do not account for the observation that ErbB4 splicing isoforms that feature cytoplasmic domain heterogeneity exhibit differential coupling to biological responses (18,27,28). For example, ectopic expression of the ErbB4 JM-a CYT-2 isoform promotes ligand-independent MCF7 breast tumor cell proliferation, though the JM-b CYT-2 isoform does not (28). Thus, ErbB4 overexpression in breast tumor samples may be the consequence of selection for ErbB4 isoforms that either fail to couple to growth arrest or act as dominant-negative mutants. A final factor to consider is that these studies do not assess whether ErbB4 overexpression predicts patient outcome independent of other factors, such as ErbB2 status. As we will discuss later, heterodimerization of ErbB2 with ErbB4 may enable ErbB4 agonists to stimulate ErbB2 coupling to cell proliferation and tumorigenesis.

Neuregulins stimulate the proliferation of various human breast and ovarian tumor cell lines. However, maximal effects are observed in cells that exhibit the highest levels of ErbB2 expression. This suggests that ErbB4-ErbB2 heterodimers, not ErbB4-ErbB4 homodimers, are coupled to proliferation in these cells (29,30). An ErbB4 ribozyme inhibits ErbB4 expression as well as neuregulin-induced anchorage-independent proliferation in the MCF7 and T47D

human breast cancer cell lines. However, because these cells exhibit endogenous ErbB2 and ErbB3 expression, the biological effects of the ErbB4 ribozyme may not be solely due to a reduction in ErbB4 expression and signaling (29).

We have highlighted two issues concerning efforts to elucidate the roles that ErbB4 plays in mammary tumorigenesis. First of all, it is still unclear whether ErbB4 is coupled to tumorigenesis or tumor suppression in mammary cells. Secondly, ErbB4 agonists and antagonists may not be the ideal tools for determining the consequences of ErbB4 signaling. ErbB4 may heterodimerize with other ErbB family receptors (31). Moreover, several ErbB4 agonists also bind other ErbB family receptors (7). In sum, ErbB4 agonists and antagonists may modulate signaling by ErbB family receptors other than ErbB4, making it difficult to ascribe their effects solely to their modulation of ErbB4 signaling.

Our goal has been to determine the effects of ErbB4 signaling on the MCF10A human mammary epithelial cell line and on a panel of human mammary tumor cell lines. To perform these experiments we have used three ErbB4 mutants (Q646C, H647C, and A648C) that are constitutively dimerized and exhibit ligand-independent phosphorylation and kinase activity (32). Thus, these mutants avoid the confounding effects of ErbB4 ligands on signaling by other ErbB family receptors. We have previously used these mutants to demonstrate that constitutive ErbB4 signaling inhibits colony formation by the DU-145 and PC-3 human prostate tumor cell lines (33). Here we demonstrate that the ErbB4-Q646C mutant markedly inhibits the formation of drug-resistant colonies by the MCF10A human mammary epithelial cell line and by the MCF7 and SKBR3 human mammary tumor cell lines. Inhibition of MCF10A colony formation by the ErbB4-Q646C mutant requires ErbB4 kinase activity. Likewise, phosphorylation of ErbB4 Tyr1056 is critical for inhibition of MCF10A colony formation by the ErbB4-Q646C mutant. Finally, inhibition of MCF10A colony formation by the ErbB4-Q646C mutant is accompanied by growth arrest rather than cell death. Taken together these data suggest that ErbB4 signaling causes tumor suppression in mammary cell lines by coupling to growth arrest. Moreover, ErbB4 kinase activity and phosphorylation of ErbB4 Tyr1056 are critical for coupling the ErbB4-Q646C mutant to tumor suppression.

MATERIALS AND METHODS

Cell Lines and Cell Culture

C127 mouse fibroblasts and the Ψ2 and PA317 recombinant retrovirus packaging cell lines were generous gifts from Dr. Daniel DiMaio (Yale University, New Haven, CT, USA). These cells were propagated as described previously (34,35). The MCF10A immortalized human mammary cell line and the T47D, MDA-MB-453, SKBR3, and MCF7 human mammary tumor cell lines were obtained from American Type Culture Collection (Manassas, VA, USA) and propagated in accordance with their recommendations. Cell culture media, supplements, and horse serum were obtained from Gibco/BRL/Life Technologies/Invitrogen (Carlsbad, CA, USA), Sigma Scientific (St. Louis, MO, USA), and Mediatech, Inc (Herndon, VA, USA). Fetal bovine serum was obtained from Gemini Bioproducts (Calabasas, CA, USA). The fetal bovine serum replacement fetal clone III was obtained from Hyclone (Logan, UT, USA). Giemsa stain was obtained from LabChem, Inc. (Pittsburgh, PA, USA). Plasticware was obtained from Becton Dickinson Labware (Franklin Lakes, NJ, USA) and Sarstedt, Inc. (Newton, NC, USA).

Plasmids and Mutants

All ErbB4 constructs described in this work are derived from the canonical JM-a, CYT-1 isoform (27,36). The recombinant retroviral expression vector pLXSN-ErbB4 and the control vector pLXSN have been described previously (31,35). We used the ErbB4 retroviral expression construct pLXSN ErbB4-Q646C, which expresses the constitutively active ErbB4-

Q646C mutant (31), for site-directed mutagenesis using the QuikChange Site Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). We created an ErbB4-Q646C mutant that lacks kinase activity (ErbB4-Q646C Kin$^-$) and phosphorylation site mutants. All of the ErbB4 mutants were validated by sequencing and by marker rescue. Primer sequences are available upon request.

To create the ErbB4-Q646C Kin$^-$ mutant we changed the Lys751 codon to a methionine codon, thereby disrupting the putative ATP binding pocket in the kinase domain. To create the ErbB4-Q646C Y1056F mutant we changed the Tyr1056 codon to a phenylalanine codon. To create the ErbB4-Q646C YChg8F-Y1056 mutant we changed the Tyr1022, Tyr1150, Tyr1162, Tyr1188, Tyr1202, Tyr1242, Tyr1258, and Tyr1284 codons to phenylalanine codons. To create the ErbB4-Q646C YChg9F mutant we used the ErbB4-Q646C YChg8F-Y1056 mutant as a template for site-directed mutagenesis and changed the Tyr1056 codon to a phenylalanine codon.

The recombinant retroviral expression plasmid pLXSN-ErbB2*, which expresses a constitutively active rat ErbB2 mutant resulting from a V664E mutation in the transmembrane domain, was the generous gift of Dr. Lisa Petti (Albany College of Medicine, Albany, NY, USA).

Generation of Recombinant Retroviruses and Retrovirus Producer Cell Lines

We generated the recombinant retrovirus stocks and retrovirus producer cell lines essentially as described previously (33,34). Briefly, we stably transfected the recombinant retroviral constructs into the Ψ2 ecotropic retrovirus packaging cell line to generate ecotropic retrovirus producer cell lines and low titer ecotropic retrovirus stocks. We stably infected the PA317 amphotropic packaging cell line with the ecotropic retrovirus stocks to generate amphotropic retrovirus producer cell lines and high titer amphotropic retrovirus stocks.

Assay Formation of Drug-Resistant Colonies on Plastic

We have developed a sensitive quantitative assay for growth inhibition of a cell line of interest by a gene of interest (33). This assay is based on one used to determine that p53 behaves as a tumor suppressor (37,38). This assay involves infecting the cell line of interest with a recombinant retrovirus that expresses the gene of interest as well as the neomycin resistance gene. We then compare the number of drug-resistant colonies that emerge from this infection with the number of drug-resistant colonies that emerge from infection of a control cell line with the same retrovirus. Infections with a vector control retrovirus serve as controls for infectivity.

We infected MCF10A, MCF7, SKBR3, MDA-MB-453, and T47D cells with the recombinant amphotropic retrovirus stocks described earlier. In every trial, C127 cells were infected in parallel using the same viral stocks as a control for viral titer. Colonies of infected cells were selected using 400–1100 μg/ml G418 and stained using Giemsa. We digitized the tissue culture plates using a UMAX Astra flatbed scanner (Dallas, TX, USA) set for 600 dpi. We manipulated the digital images using Adobe Photoshop Elements (San Jose, CA, USA).

We counted drug-resistant colonies and calculated the titer of each retroviral stock in each cell line by dividing the number of drug-resistant colonies by the volume of retrovirus used. To compare the relative efficiency of each retrovirus stock at inducing drug-resistant colony formation in the MCF10A cell line, we divided the titer of each retrovirus stock in the MCF10A cell line by the titer of the same stock in the C127 cell line. These values are expressed as mean percentages calculated from at least four independent sets of infections.

To calculate inhibition of colony formation of MCF10A cells by infection with an experimental retrovirus stock, we divided the colony formation efficiency of the experimental recombinant retrovirus stock in MCF10A cells by the colony formation efficiency of the vector control (LXSN) retrovirus stock or of the LXSN-ErbB4 retrovirus stock in MCF10A cells; we subtracted this value from 100%. We performed analogous calculations for all of the experimental retrovirus stocks following infection of the MCF10A, MCF7, SKBR3, MDA-MB-453, and T47D cell lines. These values are reported as mean percentages calculated from at least four independent sets of infections. The standard error for each mean is also reported.

Immunoprecipitation and Immunoblotting

We analyzed ErbB4 expression and tyrosine phosphorylation by anti-ErbB4 immunoprecipitation and anti-phosphotyrosine and anti-ErbB4 immunoblotting essentially as described previously (31,32,39). Briefly, PA317 cells engineered to express the ErbB4 constructs were starved in serum-free medium and then lysed in an isotonic buffer. We precipitated ErbB4 using an anti-ErbB4 rabbit polyclonal antibody (SC-283) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). We resolved the samples by SDS-PAGE and electroblotted them onto nitrocellulose. We measured ErbB4 tyrosine phosphorylation using the 4G10 anti-phosphotyrosine mouse monoclonal antibody (Upstate Biotechnology, Charlottesville, VA, USA). We measured ErbB4 expression using the SC-283 anti-ErbB4 rabbit polyclonal antibody (Santa Cruz Biotechnology). Antibody binding was visualized by enhanced chemiluminesence. We digitized the chemilumigrams using a UMAX Astra flatbed scanner and we manipulated the images using Adobe Photoshop.

Cell Fate Assay

We seeded 1×10^3 MCF10A or C127 cells in 60-mm dishes and 24 h later we marked 20 well-spaced individual cells on each plate. We immediately infected the cells with retrovirus stocks that express wild-type ErbB4 or the ErbB4-646C mutant. The multiplicity of infection was approximately six colony-forming units per cell. Cells were mock infected with PBS as a negative control. We microscopically examined each marked cell every 8–12 h for 4–8 days after infection to confirm that the cells had not migrated away from the mark. We performed four independent experiments and recorded images of representative cells by digital photomicroscopy. We manipulated the digital images using Adobe Photoshop.

Following microscopic inspection of the marked cells, we recorded whether the cell remained as a single cell (singleton), divided to form a colony, or was absent. The average number of cells with each fate is reported. The standard error for each mean is also reported. We compared MCF10A cells infected with wild-type ErbB4 to MCF10A cells infected with the ErbB4-Q646C mutant using a chi-square analysis with two degrees of freedom. We report the *p* value of this comparison. Because of the small percentage of C127 cells that are absent following retrovirus infection, we performed a Fisher's exact test (with two degrees of freedom) to compare C127 cells infected with wild-type ErbB4 to C127 cells infected with the ErbB4-Q646C mutant. We report the *p* value of this comparison.

RESULTS

The ErbB4-Q646C Mutant Specifically Inhibits Drug-Resistant Colony Formation by the MCF10A Human Mammary Cell Line and the MCF7 and SKBR3 Human Mammary Tumor Cell Lines

We have previously described Q646C, H647C, and A648C, three ErbB4 mutants that were generated by substituting a single cysteine residue for a Gln646, His647, and Ala648. These mutants display ligand-independent tyrosine kinase activity and tyrosine phosphorylation. Unlike the constitutively active V664E rat ErbB2 mutant, these ErbB4 mutants fail to

malignantly transform the mouse C127 fibroblast cell line. Thus, we concluded that ErbB4, unlike ErbB2, does not appear to be an oncogene (32).

Our current hypothesis is that ErbB4 acts as a mammary tumor suppressor. To test this hypothesis we used a quantitative assay (33) to determine the effects of the constitutively active ErbB4 mutants on the MCF10A human mammary cell line and on the SKBR3, MCF7, T47D, and MDA-MB-453 tumor cell lines. We infected cells with recombinant retroviruses that coexpress a constitutively active ErbB4 mutant along with a neomycin resistance gene and counted the number of drug-resistant colonies that arose from these infections. Infections of mouse C127 fibroblasts (which do not respond to ErbB4 signaling) and infections using a vector control retrovirus carrying only the neomycin resistance gene served as controls.

As shown in Figures 1 and 2, MCF7 and MCF10A cells infected with the ErbB4-Q646C retrovirus formed fewer drug-resistant colonies than did cells infected with the other retroviruses. Similar results were obtained from infections of the SKBR3 human mammary tumor cell line (data not shown). Indeed, the titer of the ErbB4-Q646C retrovirus was somewhat lower than the titers of most of the other retroviruses in MCF10A, MCF7, and SKBR3 cells (Table 1). In contrast, the titer of the ErbB4-Q646C retrovirus in the C127 mouse fibroblast cell line was comparable to, if not greater than, the titer of most of the other retroviruses in the C127 cells (Table 1). Thus, independent of absolute viral titer, the ErbB4-Q646C retrovirus was less capable of causing the formation of drug-resistant colonies of MCF10A, MCF7, and SKBR3 cells than were the other recombinant retroviruses. We quantified this effect by dividing the titer of the recombinant retrovirus stocks in the MCF10A, MCF7, and SKBR3 cell lines by the titer of the same retrovirus stocks in the C127 cell lines. As shown in Table 1, the efficiency of the ErbB4-Q646C retrovirus at inducing drug-resistant colonies MCF10A, MCF7, and SKBR3 cells was markedly less than the colony formation efficiency of the other retroviruses in these cells. By comparing the efficiency of the LXSN vector control retrovirus to that of the other retroviruses, we calculated that the ErbB4-Q646C retrovirus inhibited retrovirus-induced drug-resistant colony formation by 64–69% in the MCF10A, MCF7, and SKBR3 cell lines (Table 1). In contrast, wild-type ErbB4, the H647C and A648C ErbB4 mutants, and the constitutively active ErbB2 mutant did not appear to markedly inhibit drug-resistant colony formation in the MCF10A, MCF7, and SKBR3 cell lines (Table 1). These data indicate that constitutive ErbB4 signaling from the Q646C mutant is specifically coupled to inhibition of mammary cell colony formation and suggest that the loss of ErbB4 expression and signaling in mammary tumors may contribute to deregulated cell proliferation and malignancy.

However, the ErbB4-Q646C mutant did not inhibit drug-resistant colony formation by all human mammary tumor cell lines. The MDA-MB-453 and T47D cell lines formed abundant drug-resistant colonies following infection with the ErbB4-Q646C retrovirus (data not shown). Moreover, the colony formation efficiency of the ErbB4-Q646C retrovirus in these cell lines was not markedly lower than the colony formation efficiency displayed by the LXSN vector control retrovirus. Thus, the ErbB4-Q646C retrovirus did not markedly inhibit drug-resistant colony formation in these cell lines (data not shown).

ErbB4 Kinase Activity Is Required for Inhibition of Colony Formation by the ErbB4-Q646C Mutant

Next we evaluated whether ErbB4 tyrosine kinase activity is required for inhibition of colony formation by the ErbB4-Q646C mutant. We assayed the activity of an ErbB4 retrovirus that contains the Q646C activating mutation as well as a mutation (K751M) that disrupts ErbB4 tyrosine kinase activity (Q646C Kin$^-$). MCF10A and MCF7 cells infected with the Q646C Kin$^-$ retrovirus formed far more drug-resistant colonies than did cells infected with the ErbB4-Q646C virus (Figs. 1 and 2). Similar results were obtained from infections of the SKBR3

human mammary tumor cell line (data not shown). Calculations analogous to those described earlier indicated that, unlike the Q646C mutant, the Q646C Kin$^-$ mutant did not display reduced colony formation efficiency and did not appreciably inhibit colony formation (Table 1). These data suggest that ErbB4 kinase activity is required to couple the ErbB4-Q646C mutant to inhibition of mammary cell colony formation.

The Constitutively Active ErbB4 Mutants Are More Highly Expressed and Tyrosine Phosphorylated Than Is Wild-Type ErbB4

Because ErbB4 tyrosine kinase activity is required for the ErbB4-Q646C mutant to inhibit colony formation, one potential explanation for the failure of the ErbB4-H647C and A648C mutants to inhibit colony formation is that these mutants may not be stably expressed or phosphorylated. Expression of the ErbB4-Q646C mutant cannot be detected in infected MCF10A, MCF7, or SKBR3 cell lines. This is not surprising given the profound inhibitory effect this mutant has on colony formation in these cells. Thus, we evaluated the expression and tyrosine phosphorylation of the ErbB4 mutants using PA317 mouse fibroblasts infected with the ErbB4 retroviruses. Cells that express the ErbB4-H647C and ErbB4-A648C mutants displayed greater ErbB4 tyrosine phosphorylation than did the cells that express the ErbB4-Q646C mutant (Fig. 3a). Moreover, cells that express the ErbB4-H647C and ErbB4-A648C mutants displayed ErbB4 expression comparable to that displayed by the cells that express the ErbB4-Q646C mutant (Fig. 3b). Thus, the failure of the ErbB4-H647C and A648C mutants to inhibit colony formation by the MCF10A, MCF7, and SKBR3 cell lines does not appear to be due to their reduced expression or tyrosine phosphorylation.

Phosphorylation of Tyrosine 1056 Is Required and May Be Sufficient for Inhibition of MCF10A Colony Formation by the ErbB4 Q646C Mutant

Coupling of ErbB family receptors to downstream signaling effectors is in many cases dependent on interactions of the effector protein SH2 or PTB domain with a specific phosphorylated receptor tyrosine residue (1–3,5,8). We used ErbB4 phosphorylation site mutants to evaluate whether a specific site of ErbB4 phosphorylation is critical for coupling the ErbB4-Q646C mutant to inhibition of MCF10A colony formation. There are nine putative sites of ErbB4 tyrosine phosphorylation (Tyr1022, Tyr1056, Tyr1150, Tyr1162, Tyr1188, Tyr1202, Tyr1242, Tyr1258, and Tyr1284) (40–42). In the context of the ErbB4-Q646C allele we generated a mutant in which all nine of these tyrosine residues were changed to phenylalanine (Q646C YChg9F). MCF10A cells infected with this mutant formed abundant colonies (Fig. 4). Moreover, the Q646C YChg9F mutant displayed much greater colony formation efficiency than did the Q646C control and it failed to inhibit MCF10A colony formation (Table 2). Thus, at least one of the nine tyrosine residues absent in the YChg9F mutant is critical for inhibition of colony formation by the ErbB4-Q646C mutant.

Next we sought to identify this important tyrosine residue. In the context of the ErbB4-Q646C allele we generated a panel of nine mutants; in each mutant one of the nine putative sites of tyrosine phosphorylation was changed to phenylalanine. MCF10A cells infected with the mutant in which Tyr1056 was changed to phenylalanine (Q646C Y1056F) formed abundant colonies (Fig. 4). Moreover, the Q646C Y1056F mutant displayed much greater colony formation efficiency than did the Q646C control and it failed to inhibit MCF10A colony formation (Table 2). These data suggest that phosphorylation of Tyr1056 is necessary for inhibition of MCF10A colony formation by the ErbB4-Q646C mutant.

In the context of the ErbB4-Q646C allele we generated a mutant in which all of the nine putative sites of ErbB4 tyrosine phosphorylation except for Tyr1056 were mutated to phenylalanine (Q646C YChg8F-Y1056). MCF10A cells infected with this mutant formed far fewer colonies than did cells infected with the wild-type ErbB4 retrovirus (ErbB4) or cells infected with the

Q646C Y1056F retrovirus (Fig. 4). The Q646C YChg8F-Y1056 mutant displayed diminished colony formation efficiency that was comparable to that displayed by the Q646C mutant (Table 2). Moreover, the Q646C YChg8F-Y1056 mutant inhibited MCF10A colony formation to approximately the same degree as the Q646C mutant (Table 2). Thus, with regard to ErbB4 tyrosine phosphorylation, Tyr1056 appears to be sufficient for inhibition of MCF10A colony formation by the ErbB4-Q646C mutant.

Loss of Phosphorylation at Tyr1056 Does Not Destabilize the ErbB4-Q646C Mutant

One possible explanation for the failure of the ErbB4-Q646C Kin$^-$, ErbB4-Q646C YChg9F, and ErbB4-Q646C Y1056F mutants to inhibit colony formation is that disrupting ErbB4 kinase activity or eliminating ErbB4 phosphorylation at Tyr1056 destabilizes ErbB4. We assayed ErbB4 tyrosine phosphorylation (Fig. 5a) and expression (Fig. 5b) in PA317 mouse fibroblasts that express ErbB4 mutants. The two loss-of-function Q646C YChg9F and Q646C Y1056F mutants exhibited ErbB4 expression levels comparable to that of the Q646C mutant (Fig. 5b). Thus, the Y1056F mutation does not destabilize ErbB4 and is likely to disrupt inhibition of colony formation by eliminating phosphorylation-dependent binding of an effector protein to ErbB4 Tyr1056. In contrast, cells that expressed the Q646C Kin$^-$ mutant displayed dramatically less ErbB4 expression than cells that expressed the Q646C mutant (Fig. 5b). Thus, we cannot formally rule out the possibility that the failure of the Q646C Kin$^-$ mutant to inhibit mammary cell colony formation is due to the reduced expression or stability of this mutant. However, the Q646C Kin$^-$ mutant was expressed at a level comparable to that of the Q646C YChg8F-Y1056 mutant (Fig. 5b), which was capable of inhibiting colony formation. Apparently only a modest amount of expression is sufficient for inhibition of mammary cell colony formation by the ErbB4 Q646C mutant.

The Q646C Y1056F mutant did not display significantly less tyrosine phosphorylation than the Q646C mutant (Fig. 5a). This suggests that ErbB4 contains multiple sites of tyrosine phosphorylation. The Q646C YChg8F-Y1056 and Q646C Kin$^-$ mutants exhibited minimal ErbB4 tyrosine phosphorylation (Fig. 5a). However, these mutants exhibited less expression than the Q646C mutant (Fig. 5b). Thus, it is unclear that whether the eight putative sites of tyrosine phosphorylation that are absent in the YChg8F-Y1056 mutant represent bona fide sites of tyrosine phosphorylation or whether the apparent loss of phosphorylation is due to reduced expression or stability of this mutant. The Q646C YChg9F mutant displayed substantial tyrosine phosphorylation, although less phosphorylation than exhibited by the Q646C mutant (Fig. 5a). Either we did not predict all of the phosphorylation sites for the ErbB4 Q646C mutant or the absence of bona fide phosphorylation sites in the YChg9F mutant results in phosphorylation at cryptic sites. It is tempting to speculate that ErbB4 phosphorylation positively regulates ErbB4 expression or stability. Indeed, the Q646C Kin$^-$ and Q646C YChg8F-Y1056 mutants were expressed at much lower levels than the Q646C mutant (Fig. 5b). However, the level of expression of the Q646C YChg9F mutant was comparable to that of the Q646C mutant (Fig. 5b). Thus, the simple model that a single site of ErbB4 phosphorylation regulates ErbB4 stability cannot explain our data.

The ErbB4-Q646C Mutant Causes Growth Arrest Rather Than Death in MCF10A Human Mammary Cells

We hypothesized that signaling by the ErbB4 Q646C mutant inhibits colony formation by coupling to cell death or growth arrest. We tracked the fates of MCF10A or C127 cells infected with the wild-type ErbB4 or ErbB4-Q646C retroviruses. Representative photographs from each MCF10A infection are shown in Figure 6. MCF10A cells infected with the wild-type ErbB4 retrovirus proliferated and formed colonies. Most of the MCF10A cells infected with the ErbB4 Q646C retrovirus did not proliferate and form colonies and did not die and disappear

altogether. Instead, these cells appear to have undergone growth arrest. The morphology of these cells suggests that they may be undergoing autophagy (43).

The results of multiple independent experiments are shown in Table 3. A higher proportion of the MCF10A cells infected with the ErbB4-Q646C retrovirus underwent growth arrest than mock infected MCF10A cells or MCF10A cells infected with the wild-type ErbB4 retrovirus. Similarly, a much lower proportion of the MCF10A cells infected with the ErbB4-Q646C retrovirus proliferated and formed colonies than did mock infected MCF10A cells or MCF10A cells infected with the wild-type ErbB4 retrovirus. This specific coupling of the ErbB4-Q646C mutant to growth arrest was statistically significant ($p < 0.001$). Finally, the fate of C127 cells infected with the ErbB4-Q646C retrovirus was not significantly different from the fate of mock infected C127 cells or the fate of C127 cells infected with the wild-type ErbB4 retrovirus. Thus, these data indicate that the ErbB4-Q646C mutant is specifically coupled to growth arrest in MCF10A cells.

DISCUSSION

The ErbB4-Q646C Mutant Acts as a Mammary Tumor Suppressor in a Subset of Mammary Cell Lines

The ErbB4-Q646C mutant inhibits colony formation by the MCF10A human mammary cell line and by the MCF7 and SKBR3 human mammary tumor cell lines. This suggests that ErbB4 is a bona fide mammary tumor suppressor rather than just a marker for mammary cell growth arrest. However, the ErbB4-Q646C mutant fails to inhibit colony formation by the MDA-MB-453 and T47D cell lines. A potential explanation why these cells do not respond is that they may not express the ErbB4-Q646C mutant. Colonies of MDA-MB-453 and T47D cells resistant to G418 arise following infection with the ErbB4-Q646C retrovirus, indicating that the infections had been successful. However, increased ErbB4 expression is not observed in these cells, suggesting that the failure of the ErbB4-Q646C mutant to inhibit colony formation in these cells is due to inadequate ErbB4 expression. Definitive proof awaits studies in which the ErbB4-Q646C mutant is expressed from a stronger promoter.

Another explanation for the failure of the ErbB4-Q646C mutant to inhibit colony formation by the MDA-MB-453 and T47D cells may be that these cells express high levels of EGFR or ErbB2. Thus, heterodimerization of EGFR or ErbB2 with the ErbB4 Q646C mutant may inhibit homodimerization and signaling by the ErbB4 Q646C mutant. However, unpublished data from our laboratory indicate that the ErbB4 Q646C mutant does not heterodimerize with other ErbB family receptors.

A third explanation is that proteins that normally couple the ErbB4-Q646C mutant to inhibition of colony formation may not be expressed or functional in the MDA-MB-453 and T47D cells. Candidate proteins include those that have been reported to be critical for ErbB4 function. ErbB4 cleavage by tumor necrosis factor-α converting enzyme (TACE) and γ-secretase and translocation of the ErbB4 intracellular domain are required for transactivation of the β-casein promoter by STAT5a, a characteristic event in the terminal differentiation of the mammary epithelium (44–47). Preliminary data from our laboratory indicate that an ErbB4-Q646C mutant with a mutation (V673I) that abrogates cleavage by γ-secretase (47) fails to inhibit colony formation by MCF10A and MCF7 cells. Thus, the absence of γ-secretase, TACE, or STAT5a function could be responsible for the failure of ErbB4-Q646C to inhibit colony formation by the T47D and MDA-MB-453 cell lines.

Another possibility is that the ErbB4 cytoplasmic domain may interact with nuclear hormone receptors via an LXXLL motif and modulate their activity. Indeed, in MCF7 cells engineered to overexpress ErbB2, the cytoplasmic domain of ErbB2 (which contains an LXXLL motif)

binds the estrogen receptor and localizes the estrogen receptor to the plasma membrane (48). Preliminary data from our laboratory indicate that a mutation in one of the ErbB4 LXXLL motifs abrogates inhibition of MCF10A and MCF7 colony formation by the ErbB4-Q646C mutant. We hypothesize that the ErbB4-Q646C mutant functions as a nuclear hormone corepressor and that this activity is required for inhibition of colony formation by the ErbB4-Q646C mutant. Moreover, the absence of nuclear hormone receptor expression or function may account for the failure of some cell lines to respond to the ErbB4-Q646C mutant. However, because responsiveness to the ErbB4-Q646C mutant does not correlate with estrogen receptor expression (29,49), other nuclear hormone receptors may mediate ErbB4 function.

The ErbB4 cytoplasmic domain contains a putative Bcl-2 homology domain 3 (BH3) motif, which may bind proteins that regulate apoptosis (50). Ligand-induced ErbB4 cleavage and release of the ErbB4 cytoplasmic domain is followed by translocation of this domain to the mitochondria. Mutations that block ErbB4 cleavage and translocation of the ErbB4 cytoplasmic domain to the mitochondria block ligand-induced ErbB4 coupling to apoptosis (47). Moreover, preliminary data from our laboratory indicate that mutations in the BH3 domain abrogate inhibition of MCF7 and MCF10A colony formation by the ErbB4-Q646C mutant. Thus, we hypothesize that the ErbB4-Q646C mutant inhibits colony formation by binding proapoptotic proteins and stimulating their function and that cells resistant to the effects of the ErbB4-Q646C mutant lack these proapoptotic proteins.

Finally, it is possible that the MDA-MB-453 and T47D cells may overexpress or have acquired activating mutations in oncogenes that make these cells resistant to the ErbB4-Q646C mutant. Overexpression of EGFR or ErbB2 correlates with resistance to chemotherapeutic agents and radiation therapy (51). Thus, overexpression of EGFR or ErbB2 may abrogate growth inhibition by the ErbB4-Q646C mutant. Unfortunately, responsiveness to the ErbB4-Q646C mutant does not correlate with a pattern of ErbB family receptor expression (49,51). Similarly, increased phosphatidylinositol 3′ kinase (PI3 kinase) activity may be involved in resistance to the ErbB4-Q646C mutant. Overexpression of PI3 kinase or activating mutations in PI3 kinase that cause increased levels of phosphorylated Akt may lead to chemoresistance or radioresistance in breast cancer cells (52,53). Thus, these events may also correlate with resistance to inhibition of colony formation by the ErbB4-Q646C mutant.

Phosphorylation of ErbB4 on Y1056 Is Critical for ErbB4 Coupling

Here we demonstrate that ErbB4 kinase activity is required for inhibition of colony formation by ErbB4, suggesting that phosphorylation of ErbB4 itself is critical for inhibition of colony formation. Mutational analyses of the putative sites of tyrosine phosphorylation in *C. elegans* LET-23 (the EGFR homolog) and rat ErbB2 have identified individual sites of tyrosine phosphorylation that are necessary and sufficient to couple these receptors to biological events (54,55). Results of an analogous approach described here indicate that phosphorylation of ErbB4 Tyr1056 is necessary and possibly sufficient for inhibition of colony formation by ErbB4.

The canonical ErbB4 CYT-1 splicing isoform is the basis for the ErbB4 mutants described here. Thus, our observation that the Tyr1056 residue is critical for coupling ErbB4 to inhibition of colony formation is consistent with the observation that the CYT-1 and CYT-2 isoforms may display differential coupling to cellular growth control (18,27,28). Indeed, preliminary data from our laboratory indicate that the Q646C mutant in the context of the CYT-2 isoform fails to inhibit colony formation by DU-145 and PC-3 human prostate tumor cell lines. Thus, the functional differences between the CYT-1 and CYT-2 splicing isoforms may be largely due to the absence or presence of the Tyr1056 phosphorylation site.

A number of effectors may be responsible for coupling ErbB4 phosphorylation at Tyr1056 to inhibition of colony formation. Phosphorylation of ErbB4 Tyr1056 permits ErbB4 association with the regulatory subunit of PI3 kinase (27,41,56). Regulation of Akt phosphorylation by PI3 kinase can regulate apoptosis (52). Thus, ErbB4-Q646C phosphorylated on Tyr1056 may bind the PI3 kinase regulatory subunit and prevent PI3 kinase signaling by mislocalizing or sequestering the PI3 kinase regulatory subunit. STAT5a is also an important effector of ligand-induced ErbB4 signaling. Phosphorylation of Tyr1056 in the ErbB4-Q646C mutant may permit Stat5a binding to ErbB4, enabling STAT5a tyrosine phosphorylation, STAT5a dimerization and STAT5a-dependent stimulation of gene expression (46,47).

A final possibility is that phosphorylation of Tyr1056 may be required for proteolytic processing of the ErbB4 protein by TACE and γ-secretase and for subsequent translocation of the ErbB4 cytoplasmic domain to the nucleus or mitochondria. As we have discussed earlier, translocation of the ErbB4 cytoplasmic domain may alter the localization and activity of apoptotic proteins, STAT5a, PI3 kinase, or steroid hormone receptors.

The ErbB4-Q646C Mutant Inhibits Colony Formation by Various Human Mammary Cell Lines but the ErbB4-H647C and ErbB4-A648C Mutants Do Not

Despite the fact that the ErbB4-H647C and A648C mutants are constitutively dimerized and tyrosine phosphorylated (32), they fail to inhibit colony formation by the MCF10A, MCF7, and SKBR3 cell lines. These results echo our observation that the ErbB4-H647C and A648C mutants fail to inhibit colony formation by the DU-145 and PC-3 human prostate tumor cell lines (33).

There are two potential reasons why the ErbB4-H647C and A648C mutants fail to inhibit colony formation. One possibility is that the ErbB4-Q646C mutant is phosphorylated on different tyrosine residues than are the H647C and A648C mutants. Specifically, the Q646C mutant may be phosphorylated at Tyr1056 but the H647C and A648C mutants may not be. However, preliminary data from our laboratory suggest that the ErbB4-Q646C, ErbB4-H647C, and ErbB4-A648C mutants are all phosphorylated at Tyr1056. Nonetheless, the ErbB4-Q646C YChg9F mutant is still highly phosphorylated on tyrosine residues, indicating that there may be additional bona fide sites of ErbB4 tyrosine phosphorylation. These phosphorylation sites may be necessary for inhibition of colony formation and these sites may not be phosphorylated in the H647C and A648C mutants.

Another possibility is that the Q646C, H647C, and A648C mutations cause differential juxtapositioning of the ErbB4 monomers within the ErbB4 dimer. Substitution of a cysteine residue for Val656 and Thr657 in the extracellular juxtamembrane domain of rat ErbB2 results in a mutant that malignantly transforms rat fibroblasts. However, substitution of a cysteine residue at other locations in the extracellular juxtamembrane domain of rat ErbB2 fails to cause transforming activity (57). Similarly, small deletions in the extracellular juxtamembrane domain of rat ErbB2 that include a cysteine residue can in some instances yield mutants with transforming activity (58). Whereas creation of an intermolecular disulfide linkage in the extracellular juxtamembrane region of ErbB2 can in some cases yield transforming activity, differential juxtapositioning of the receptor monomers may account for the specificity of the activating mutations.

Indeed, the location of ErbB2 intermolecular disulfide linkages capable of yielding transforming activity exhibits periodicity, suggesting that juxtaposing a specific face of two receptor monomers is required to yield a receptor with transforming activity. Circular dichroism studies reveal that the ErbB2 extracellular juxtamembrane region is alpha helical (59). Thus, differential rotational juxtapositioning of the ErbB2 and ErbB4 monomers could

define receptor coupling to biological responses by regulating access of signaling effectors to the receptor tyrosine kinase domain or to sites of receptor tyrosine phosphorylation.

This proposed mechanism for signaling specificity does not appear to be restricted to the constitutively active ErbB4 mutants. It has been suggested that different ligands for the same ErbB family receptor lead to subtle differences in the conformation of the receptor dimer (60), thereby accounting for differential ligand-induced EGFR signaling (61) and differential ligand-induced ErbB4 signaling (62–65).

ErbB4 Causes Tumor Suppression by Inducing Cellular Growth Arrest

MCF10A cells that express the ErbB4-Q646C mutant remain as single, growth-arrested cells but do not die. However, preliminary analyses using Oil Red staining indicate that the ErbB4-Q646C mutant does not induce differentiation of MCF10A cells. Thus, ErbB4 signaling is coupled to growth arrest but not terminal differentiation in normal human mammary cells. This conclusion is consistent with the observation that ErbB4 expression in the mouse mammary epithelium is elevated during a period late in pregnancy characterized by reduced epithelial cell proliferation (22). Thus, proteins that couple to epithelial cell growth arrest may be ErbB4 effectors.

It is possible the ErbB4-Q646C mutant will trigger apoptosis rather than growth arrest in human mammary tumor cell lines. Such a finding would be consistent with the observation that ErbB4 agonists trigger apoptosis in human mammary tumor cell lines (66) and with our observation that the ErbB4 BH3 motif is critical for inhibition of colony formation. Such a finding would also suggest that the ErbB4 effector proteins in MCF10A and other nonmalignant mammary cells may be distinct from those found in human mammary tumor cell lines.

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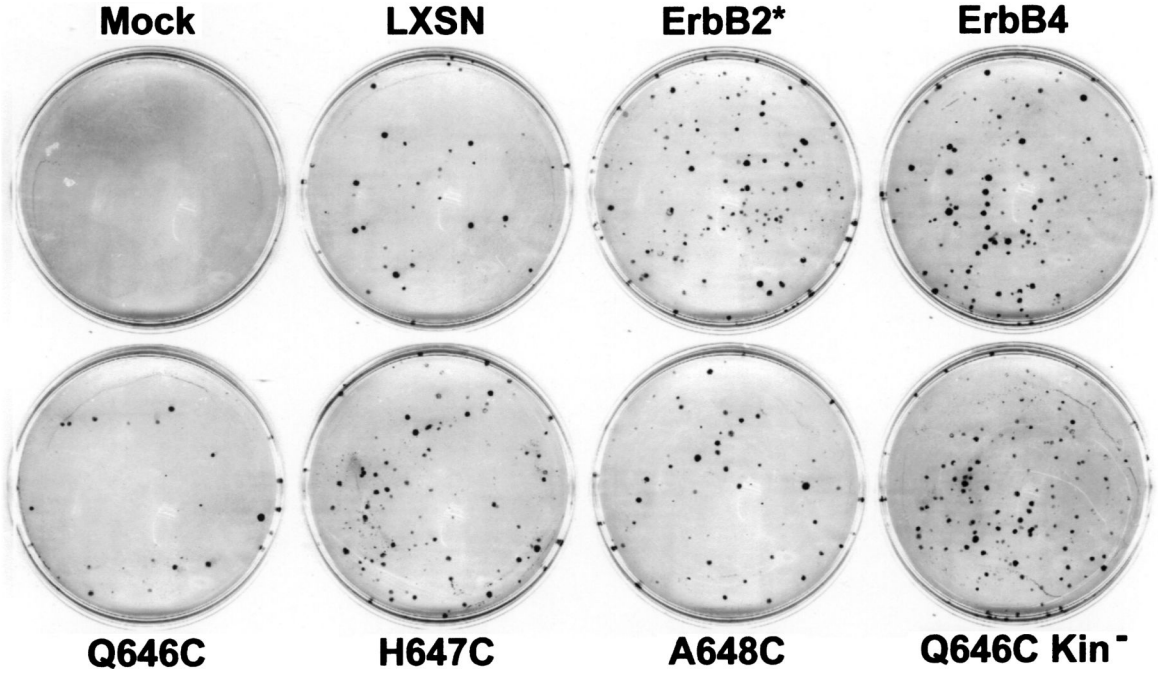

Figure 1.

The ErbB4-Q646C mutant inhibits drug-resistant colony formation by the MCF7 human mammary tumor cell line and this inhibition is dependent on ErbB4 kinase activity. MCF7 cells were infected with recombinant amphotropic retroviruses that express the neomycin resistance gene along with a constitutively active ErbB2 mutant (ErbB2*), wild-type ErbB4 (ErbB4), constitutively active ErbB4 mutants (Q646C, H647C, A648C), or an ErbB4 Q646C allele that lacks tyrosine kinase activity (ErbB4-Q646C Kin^{-}). Cells were also infected with a vector control recombinant retrovirus that expresses only the neomycin resistance gene (LXSN). Infected cells were selected using G418 and colonies of drug-resistant cells were stained using Giemsa and counted. Representative culture dishes were digitized and are shown.

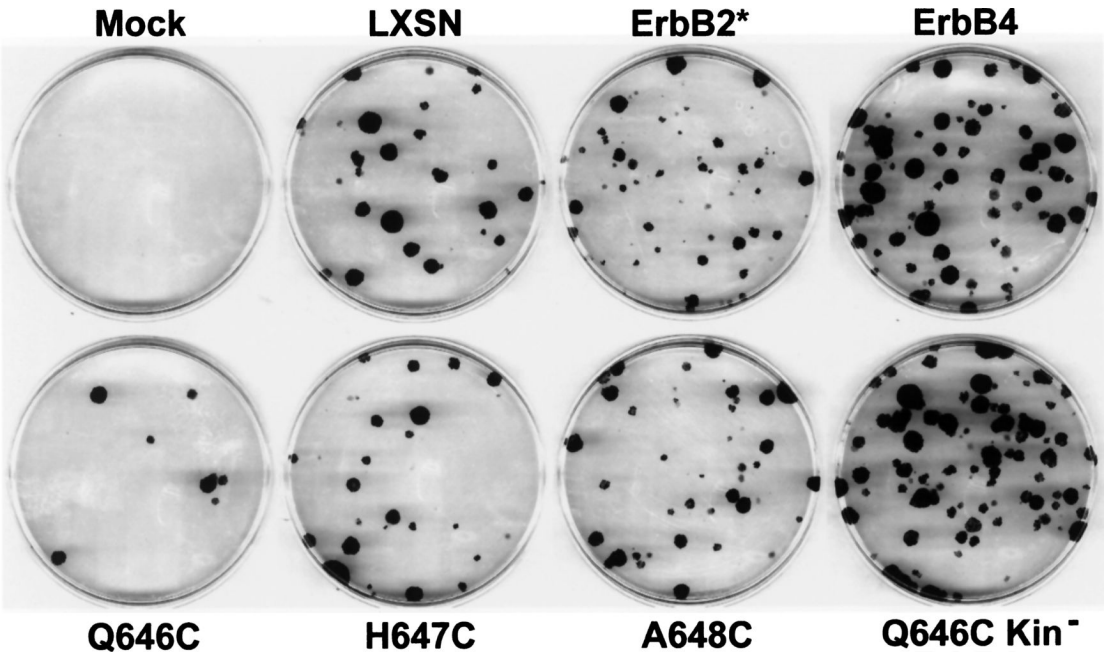

Figure 2.

The ErbB4-Q646C mutant inhibits drug-resistant colony formation by the MCF10A human mammary cell line and this inhibition is dependent on ErbB4 kinase activity. MCF10A cells were infected with recombinant amphotropic retroviruses that express the neomycin resistance gene along with a constitutively active ErbB2 mutant (ErbB2*), wild-type ErbB4 (ErbB4), constitutively active ErbB4 mutants (Q646C, H647C, A648C), or an ErbB4 Q646C allele that lacks tyrosine kinase activity (ErbB4-Q646C Kin^{-}). Cells were also infected with a vector control recombinant retrovirus that expresses only the neomycin resistance gene (LXSN). Infected cells were selected using G418 and colonies of drug-resistant cells were stained using Giemsa and counted. Representative culture dishes were digitized and are shown.

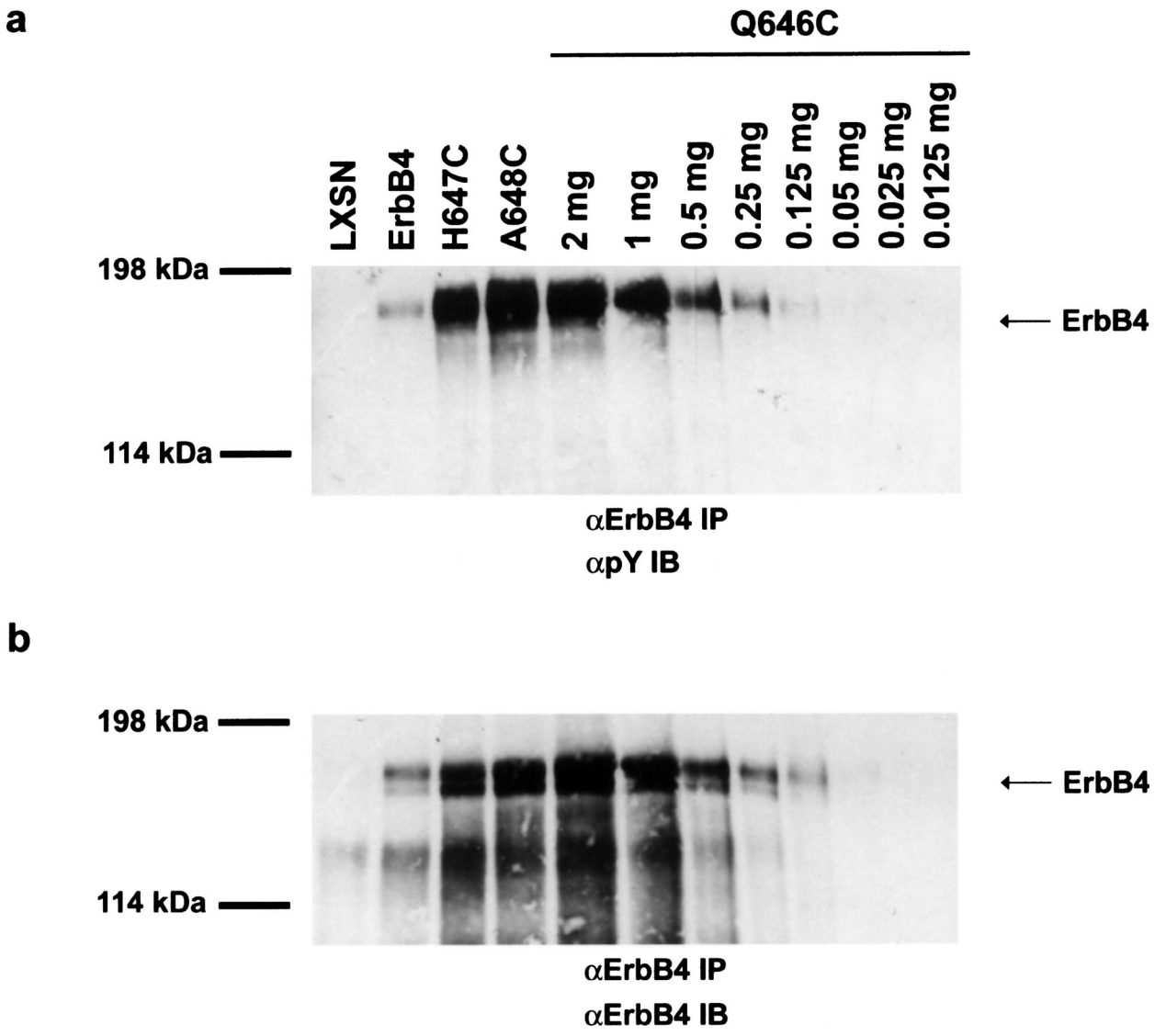

Figure 3.

The constitutively active ErbB4 mutants are more highly expressed and tyrosine phosphorylated than is wild-type ErbB4. ErbB4 expression and tyrosine phosphorylation were evaluated by immunoprecipitation and Western blotting as described elsewhere using lysates prepared from PA317 cells infected with the retroviruses that express wild-type ErbB4 (ErbB4) or the ErbB4 mutants (H647C, A648C, Q646C). PA317 cells infected with the vector control retrovirus (LXSN) served as a negative control. ErbB4 was immunoprecipitated from 0.0125 to 2 mg of lysate prepared from the cell line that expresses the Q646C mutant and from 0.5 mg of lysate prepared from each of the other cell lines. (a) The resulting blot was probed with an anti-phosphotyrosine antibody. (b) The blot was then stripped and probed with an anti-ErbB4 antibody. The position and the reported molecular weight of the protein molecular weight standards are indicated. The position of ErbB4 on the blots is also indicated.

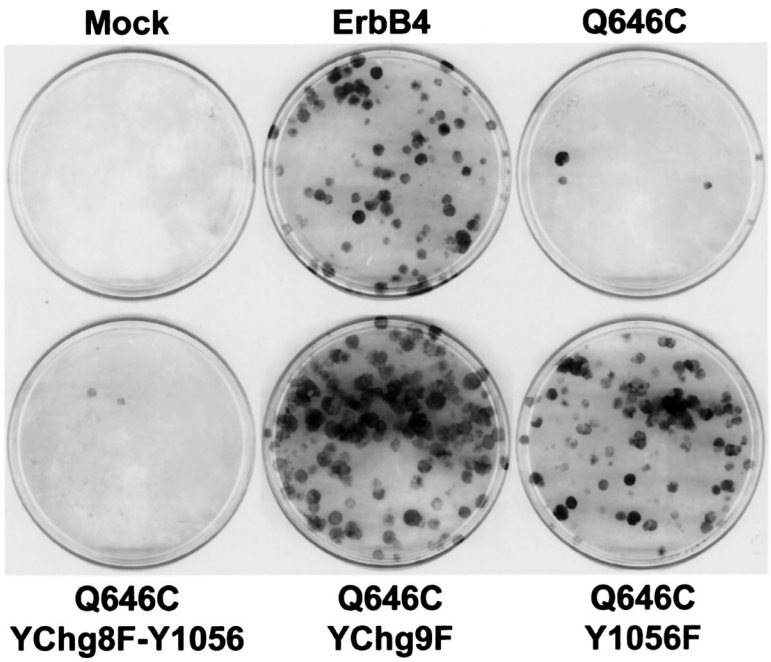

Figure 4.

Phosphorylation of ErbB4 tyrosine 1056 is critical for inhibition of colony formation by the ErbB4-Q646C mutant. MCF10A cells were infected with recombinant amphotropic retroviruses that carry the neomycin resistance gene along with wild-type ErbB4 (ErbB4), a constitutively active ErbB4 mutant (ErbB4-Q646C), or a Q646C mutant in which one or more possible sites of tyrosine phosphorylation are mutated to phenylalanine (YChg8F-Y1056, YChg9F, Y1056F). Infected cells were selected using G418 and colonies of drug-resistant cells were stained using Giemsa and counted. Representative culture dishes were digitized and are shown.

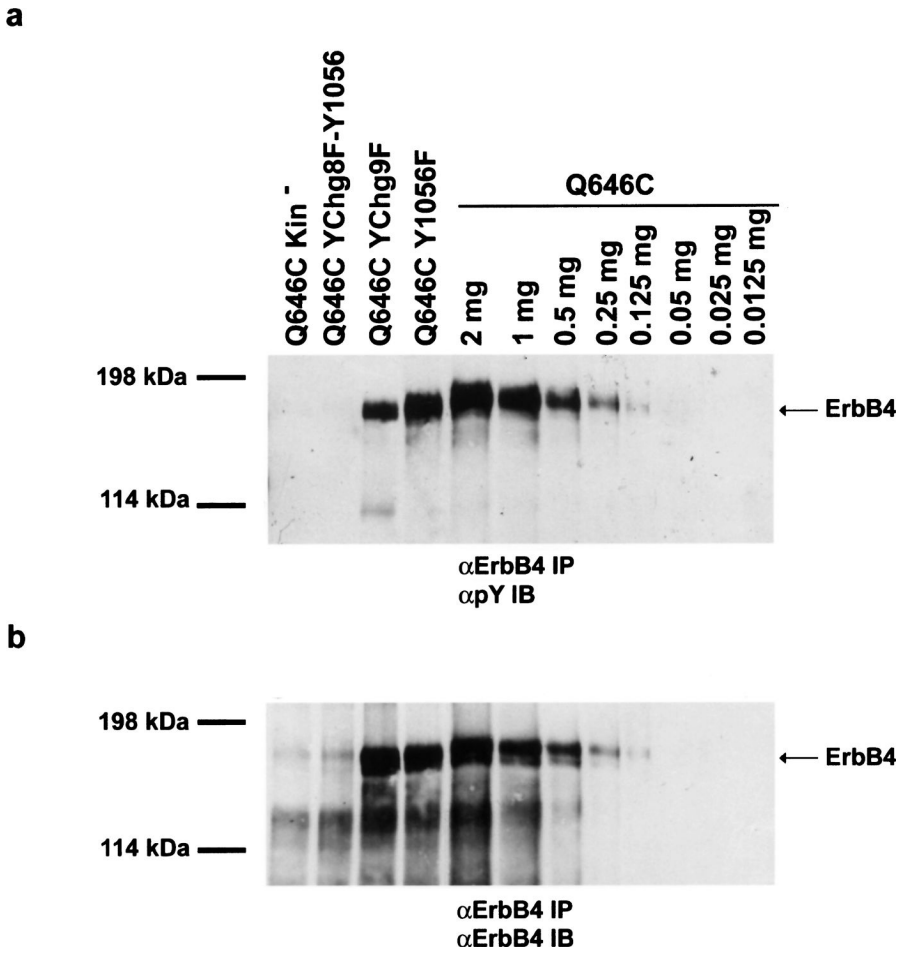

Figure 5.

Phosphorylation and expression of ErbB4-Q646C Kin⁻ and ErbB4-Q646C YChg8F-Y1056 is greatly reduced compared to ErbB4-Q646C, ErbB4-Q646C YChg9F, and ErbB4-Q646C Y1056F. ErbB4 expression and tyrosine phosphorylation were evaluated by immunoprecipitation and Western blotting as described elsewhere using lysates prepared from PA317 cells infected with the retroviruses that express the following ErbB4 mutants: Q646C, Q646C Kin⁻, Q646C YChg8F-Y1056, and Q646C YChg9F. ErbB4 was immunoprecipitated from 0.0125 to 2 mg of lysate prepared from the cell line that expresses the Q646C mutant and from 0.5 mg of lysate prepared from each of the other cell lines. (a) The resulting blot was probed with an anti-phosphotyrosine antibody. (b) The blot was then stripped and probed with an anti-ErbB4 antibody. The position and the reported molecular weight of the protein molecular weight standards are indicated. The position of ErbB4 on the blots is also indicated.

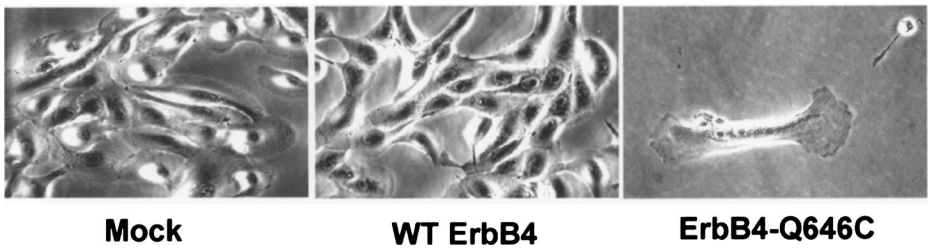

Figure 6.

The ErbB4-Q646C mutant causes growth arrest rather than death in MCF10A human mammary cells. MCF10A or C127 cells were seeded in a 60-mm dish at 1×10^3 cells/plate. Twenty cells on each plate were circled and the cells were infected with a retrovirus that directs the expression of wild-type ErbB4 or with a retrovirus that directs the expression of the ErbB4-646C mutant. Cells were mock infected as a negative control. We photographed marked cells for 4–8 days postinfection. Representative photographs are shown.

Table 1

The ErbB4-Q646C Mutant Inhibits Drug-Resistant Colony Formation by the MCF10A Human Mammary Cell Line and the MCF7 and SKBR3 Human Mammary Tumor Cell Lines

Cell Line/Infection	Retrovirus Titer	Colony Formation Efficiency (%)	Inhibition of Colony Formation (%)
C127			
LXSN	7.41E+05	100	
ErbB2*	3.22E+05	100	
ErbB4	2.03E+05	100	
Q646C	7.18E+05	100	
H647C	9.79E+05	100	
A648C	2.41E+05	100	
Q646C Kin$^-$	4.96E+05	100	
MCF10A			
LXSN	3.85E+04	5.1	
ErbB2*	1.63E+04	5.1	2 ± 5
ErbB4	1.22E+04	5.5	none
Q646C	1.43E+04	1.7	64 ± 10
H647C	7.10E+04	7.6	none
A648C	2.17E+04	8.6	none
Q646C Kin$^-$	3.20E+04	6.5	none
MCF7			
LXSN	6.89E+04	9.7	
ErbB2*	4.15E+04	15	none
ErbB4	2.49E+04	15	none
Q646C	2.01E+04	3.0	68 ± 5
H647C	8.01E+04	9.6	none
A648C	2.91E+04	13	none
Q646C Kin$^-$	5.92E+04	15	none
SKBR3			
LXSN	5.64E+04	7.3	
ErbB2*	2.44E+04	7.0	none
ErbB4	1.51E+04	8.1	none
Q646C	1.60E+04	2.2	69 ± 4
H647C	5.21E+04	5.5	23 ± 5
A648C	1.75E+04	6.8	2 ± 10
Q646C Kin$^-$	4.05E+04	9.1	none

The absolute value for 100% is 1.0.

Table 2

Phosphorylation of ErbB4 Tyr1056 Appears to be Necessary and Sufficient for Inhibition of Colony Formation by the ErbB4-Q646C Mutant

Cell Line/Infection	Retrovirus Titer	Colony Formation Efficiency (%)	Inhibition of Colony Formation (%)
C127			
ErbB4	3.18E+05	100	
Q646C	6.82E+05	100	
Q646C YChg8F-Y1056	1.63E+05	100	
Q646C YChg9F	7.41E+05	100	
Q646C Y1056F	4.31E+05	100	
MCF10A			
ErbB4	2.38E+04	11	
Q646C	1.02E+04	1.9	83 ± 2
Q646C YChg8F-Y1056	1.57E+04	1.2	88 ± 2
Q646C YChg9F	4.98E+04	8.9	4 ± 16
Q646C Y1056F	5.36E+04	14	none

The absolute value for 100% is 1.0.

Table 3

The ErbB4-Q646C Mutant Causes Cellular Growth Arrest Rather Than Cellular Death in MCF10A Human Mammary Cells

Cell Line/Virus	Cell Fate Following Infection		
	Singleton	Absent	Colony
C127			
Mock	3.0 ± 0.9	0.0 ± 0.0	17.0 ± 0.9
ErbB4	5.0 ± 0.6	0.5 ± 0.3	14.5 ± 0.6
Q646C	5.5 ± 1.0	0.0 ± 0.0	14.5 ± 1.0
ErbB4 vs. Q646C: $p = 0.530$			
MCF10A			
Mock	6.0 ± 1.4	2.3 ± 1.1	11.8 ± 2.1
ErbB4	4.5 ± 1.6	2.8 ± 0.8	12.8 ± 2.1
Q646C	13.3 ± 0.9	1.3 ± 0.8	5.5 ± 1.2
ErbB4 vs. Q646C: $p < 0.001$			