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Overexpression of ErbB2 Impairs Ligand-Dependent Downregulation of Epidermal Growth Factor Receptors Via a Post-transcriptional Mechanism

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Abstract The mechanism by which ErbB2 exerts its oncogenic effect is poorly defined. In this article we show that ErbB2 co-expression slows ligand-dependent growth factor receptor downregulation in NIH 3T3 transfectants. Ligand dependence of cell growth and MAP kinase signaling are retained in epidermal growth factor receptor (EGFR) transfectants but are abolished in ErbB2-expressing cells, which grow and signal constitutively. In association with this phenomenon, we have noticed that ErbB2-expressing cells contain increased amounts of EGFR, which is hyperphosphorylated. EGFR overexpressors do not contain increased levels of ErbB2, however, tending to exclude a transfection artifact caused by saturation of receptor processing. EGF treatment of EGFR transfectants results in more rapid EGFR downregulation than occurs in ErbB2 transfectants, but Northern blot analysis demonstrates reduced basal EGFR gene expression in ErbB2 transfectants. We conclude that ErbB2 expression impairs EGFR downregulation via a post-transcriptional mechanism and propose that ErbB2 overexpression may sensitize tumor cells to the mitogenic effects of heterologous growth factors by retarding degradation of liganded heterodimers. J. Cell. Biochem. 74:23–30, 1999.

Key words: ErbB2; EGF receptor; growth substances; downregulation

ErbB2 is a receptor-like tyrosine kinase that transforms cells when overexpressed [Di Fiore et al., 1987; Di Marco et al., 1990] or mutated [Bargmann et al., 1986]. The significance of ErbB2 overexpression in early-stage human tumors remains unclear [Allred et al., 1992], however, and activating mutations are rare [Lemoine et al., 1990]. These observations cast doubt on the assumption that ErbB2 is an independent signalling oncoprotein and, indeed, no soluble ligand has yet been confirmed. Of note, however, ErbB2 readily heterodimerizes with other type I receptor tyrosine kinases—particularly the epidermal growth factor receptor (EGFR) and ErbB3 [Kokai et al., 1989], suggesting an affinity for oligomer formation mediated by intracellular domain interactions [Kwatra et al., 1992; Chantry, 1995]. EGF-dependent heterodimerization of EGFR and ErbB2 induces ErbB2 transphosphorylation [King et al., 1988], but few other consequences of heterodimerization have been established.

In addition to ligand availability and heterologous receptor interactions, growth factor functioning is regulated by negative feedback loops that terminate receptor signaling. A key mechanism for terminating growth factor receptor signaling is downregulation—the process by which liganded receptors are degraded [Stoscheck and Carpenter, 1984] via either proteasomal [Galcheva-Gargova et al., 1995; Mori et al., 1995; Mimnaugh et al., 1996] or lysosomal

Abbreviations used: EGFR, epidermal growth factor receptor; ErbB2, HER-2/*neu;* PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MAP, mitogen-activated protein; ALP, alkaline phosphatase; TGF-α, transforming growth factor-α.

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[Hoffman et al., 1994; Opresko et al., 1995; Kornilova et al., 1996] pathways. Ligand-dependent homodimerization of growth factor receptors is generally followed by kinase activation, endocytosis into clathrin-coated pits, microtubular transport from submembranous early endosomes to perinuclear late endosomes (also termed "prelysosomal compartments" with multivesicular bodies), and phosphotyrosine-dependent routing either to degradative lysosomes or to a recycling pathway [Felder et al., 1990]. Intracellular protein routing "decisions" such as recycling may either occur by default, or may reflect vectorial interactions involving molecular filters such as those within the coated endocytic pits of the plasma membrane or the multivesicular bodies of late endosomes [Hopkins, 1992; Sorkin et al., 1996]. Organellar filters of this type recognize receptor-bound sorting signals including nexins [Kurten et al., 1996] and the ~100-kDa adaptins [Robinson, 1987] of the clathrin-binding AP-2 complex [Sorkin et al., 1993, 1995]. Potential EGFR binding sites for such sorting proteins include the C-terminal tyrosine autophosphorylation sites [Decker et al., 1992; Nesterov et al., 1995], which are implicated in receptor endocytosis and downregulation [Helin and Beguinot, 1991; Huang et al., 1997]. Both internalization and lysosomal targeting require phosphotyrosine-dependent receptor retention within the endocytic apparatus [Glenney et al., 1988; Lamaze and Schmid, 1995], although distinct signals appear to regulate these two steps [French et al., 1994; Warren et al., 1997]. Variations in such signals may explain the divergent behavior of receptors such as those for platelet-derived growth factor (PDGF) or insulin, which preferentially downregulate or recycle, respectively [Ulrich and Schlessinger, 1990].

Dysregulated growth factor signal termination caused by receptor downregulatory defects has been linked to neoplastic cell transformation [Wells et al., 1990; Pandit et al., 1996]. This raises the possibility that hetero-oligomeric interactions of liganded receptors with ErbB2, which itself appears to be endocytosis-defective [Baulida et al., 1996], could sensitize tumor cells to the mitogenic action of ambient growth factors. This report examines the effect of ErbB2 expression on EGF-dependent EGFR persistence and signaling.

MATERIALS AND METHODS

Cell Culture, Transfection, Treatment, and Growth Assays

Parental NIH 3T3 cells were obtained from the American Type Culture Collection (ATCC) and maintained in a humidified 5% CO_2 incubator at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), glutamine, and antibiotics. Cells were transfected using a calcium phosphate protocol [Chen and Okayama, 1987]. The vector used was the CVN mammalian protein expression vector, using the SV40 promoter. Six individual clones were pooled for both the EGFR and ErbB2 constructs, and transformants were isolated using G418 selection. Samples were treated with EGF (Sigma Chemical Co., St. Louis, MO) for the designated duration and at the noted concentration. Growth curves were derived using a sulphorhodamine B spectrophotometric proliferation assay and quadruplicate samples [Skehan et al., 1990]. Mitogen-activated protein (MAP) kinase activity was measured using a commercial phospho-MAP kinase antibody kit (New England Biolabs, Boston, MA).

Immunological Reagents

The pAb-1 rabbit polyclonal antibody to the tyrosine-1248-containing C-terminal peptide sequence of ErbB2 (Triton Biosciences, Alameda, CA) was reconstituted in water and diluted 1:1,000 in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) for immunoblotting. The EGFR monoclonal antibody (clone F4) was obtained from Sigma and used for immunoblotting, while for denaturing immunoprecipitations a human-specific EGFR monoclonal antibody (clone 13) was sourced from Transduction Laboratories.

Protein Lysis, Immunoblotting and Immunoprecipitation

For immunoprecipitations, 100-µl lysates were incubated with antibody for 1 h, followed by a further 30-min incubation with 40-µl protein-A/Sepharose CL4B beads (Sigma) before washing three times in ice-cold TBS, addition of sample buffer, and boiling. After immunoprecipitation, in vitro alkaline phosphatase (ALP) reactions were carried out as described using bovine ALP (AP; Sigma) [Epstein et al., 1990].

Immunoprecipitated samples were electrophoresed as above, then transblotted onto nitro-

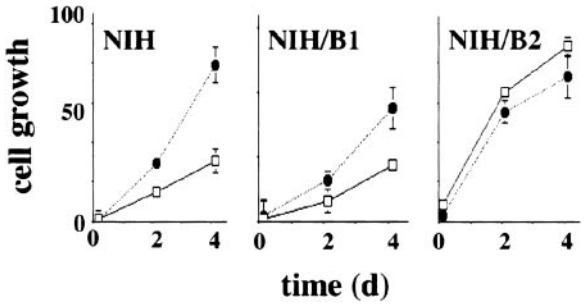

Fig. 1. Growth factor dependence of NIH 3T3 cell growth in epidermal growth factor receptor (EGFR) and ErbB2 transfectants. □, controls; ●, EGF 100 ng/ml. NIH, parental NIH 3T3 cells; NIH/B1, NIH 3T3 cells stably transfected with EGFR; NIH/B2, NIH 3T3 cells stably transfected with ErbB2. Error bars represent standard errors based on quadruplicate measurements.

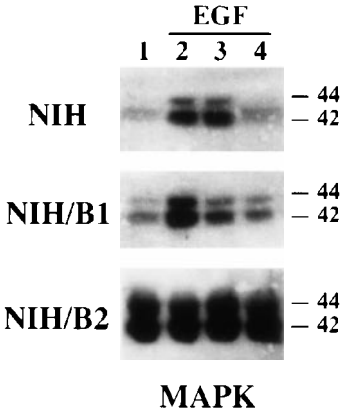

Fig. 2. Growth factor dependence of mitogen-activated protein (MAP) kinase induction in epidermal growth factor receptor (EGFR) and ErbB2 transfectants. Effects of Erb B2 expression on the kinetics of EGF-dependent MAP kinase activation in EGFR (NIH/B1) and ErbB2 (NIH/B2) 3T3 transfectants were measured using a phospho-MAP kinase antibody kit. Data from one of two similar experiments. **Lane 1,** controls; **lane 2,** EGF 100 ng/ml 1 h; **lane 3,** EGF 2 h; **lane 4,** EGF 4 h.

cellulose as described [Towbin et al., 1979]. Membranes were blocked using 2% casein for 1 h at room temperature, then incubated with primary antibody overnight at 4°C with gentle shaking. After three washes with TBST, membranes were incubated with peroxidase-conjugated (Amersham) polyclonal anti-rabbit/mouse IgG, and then developed immediately using standard chemiluminescent procedures. For immunoblotting, 50 µg of cell protein lysate was added to each well.

Nucleic Acid Blotting and Metabolic Labeling

For Northern blotting, RNA was prepared using RNAsol B (Biogenesis), while for Southern blotting, genomic DNA was isolated using a kit (Nucleon Biosciences), digested with *Hin*dIII, and the probe labeled with random hexanucleotide primers (MBI, Fermentas); blotting was carried out using standard procedures. ^{35}S-methionine protein radiolabeling was carried out as described previously [Epstein, 1995].

RESULTS

ErbB2 transfectants exhibit EGF-independent growth, whereas EGFR transfectants remain sensitive to the addition of EGF (Fig. 1). The plot shows exponential growth on a linear scale for controls and EGFR (B1) transfectants, but a growth plateau suggestive of density inhibition or starvation for the more rapidly proliferating ErbB2 transfectants. ErbB2 transfectants also display constitutive MAP kinase signaling, but MAP kinase activity in EGFR transfectants remains both ligand-inducible and transient (Fig. 2). While these observations are consistent with previous reports relating to ErbB2-dependent cell transformation, they strongly suggest a qualitative difference in the function of the EGFR and ErbB2 receptors.

To address this phenomenon, we first examined the effects of ErbB2 expression on the regulation and function of EGFR. Figure 3A shows that ErbB2 (B2) expression leads to EGFR (B1) upregulation, but that the converse does not apply (upper panels), tending to exclude the nonspecific possibility that saturation of the lysosomal pathway by receptor overexpression [French et al., 1994] accounts for upregulation of endogenous receptors by ErbB2. Furthermore, ErbB2 (B2) co-expression slows the electrophoretic mobility of EGFR to ~175 kDa (Fig. 3B, lane 2), as compared with 170-kDa EGFR-only 3T3 transfectants (B1; lane 1). This apparent increase in molecular weight is likely to reflect increased EGFR phosphorylation, since alkaline phosphatase incubation of EGFR immunoprecipitated from ErbB2-transfected cells (AP B2; lane 3) is associated with full reversion of electrophoretic mobility (Fig. 3B).

EGFR receptor half-life is normally reduced from ~10 h to 1–2 h by ligand activation [Stoscheck and Carpenter, 1984]. However, analyses of EGFR-overexpressing A431 squamous carcinoma cells have indicated the presence of "low-affinity" EGFR subsets characterized by slow ligand- and antibody-dependent downregula-

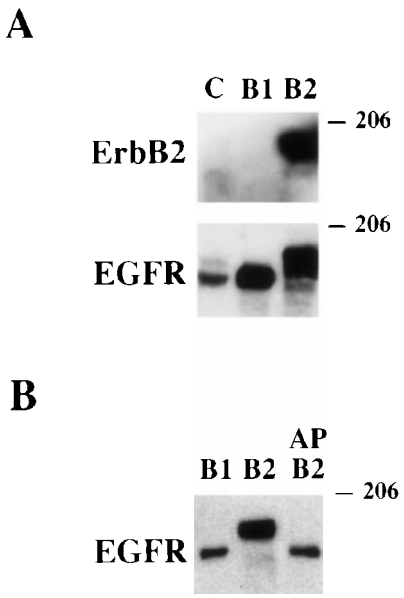

Fig. 3. Effects of ErbB2 overexpression on epidermal growth factor receptor (EGFR) in NIH 3T3 cell transfectants. **A:** Relative expression levels of EGFR and ErbB2 in ErbB2- and EGFR-transfected NIH 3T3 cells. Parental cells (C; **lane 1**) were stably transfected with either EGFR (B1; **lane 2**) or ErbB2 (B2: **lane 3**). Protein lysates were immunoblotted with antibodies to EGFR or ErbB2 (left). The results are from one of three similar experiments. **B:** Effects of ErbB2 on EGFR electrophoretic mobility in the presence and absence of phosphatases. Human EGFR transfectants (B1; **lane 1**) show the expected 170-kDa receptor size on immunoblotting; one of two similar experiments is shown. EGFR immunoprecipitates from cells transfected with human ErbB2 (**lanes 2, 3**) were suspended in alkaline phosphatase (AP) buffer, with the enzyme added only to the lane 3 immunoprecipitate (AP B2) as described [Epstein et al., 1990] before electrophoresis and immunoblotting.

tion [Kawamoto et al., 1983]. As functional receptor subsets of this type could reflect the existence of heterodimers such as we have previously demonstrated between EGFR and ErbB2 [Gulliford et al., 1997; Huang et al., 1998; Ouyang et al., 1998], we compared the durability of EGF-dependent EGFR expression with that of EGFR expression in ErbB2-transfected 3T3 cells. Figure 4A shows that EGFR transfectants (NIH/B1) downregulate EGFR normally in response to ligand, whereas ErbB2 transfectants (NIH/B2) exhibit markedly prolonged EGFR expression. Parallel immunoblots of ErbB2 and EGFR (Fig. 4B) confirm that the EGFR downregulation seen after 12- to 24-h EGF treatment is not accompanied by ErbB2 downregulation; relevant to Figure 3, this result confirms lack of cross-reactivity between the EGFR and ErbB2 antibodies. Metabolic labelling experiments were then conducted to determine the rates of EGFR turnover in EGFR and ErbB2 transfectants. Figure 5 confirms that EGFR turnover is selectively attenuated in ErbB2 transfectants.

To confirm the post-transcriptional nature of this ErbB2-dependent impairment of EGFR downregulation, Northern blot analysis of ErbB2-overexpressing cells was undertaken. Figure 6 verifies that ErbB2 transfectants express significantly less of the 5-kb EGFR mRNA transcript than do parental 3T3 cells, excluding a confounding effect of ErbB2 transfection on EGFR gene expression and implying that EGFR upregulation does not occur via increased gene transcription. Southern blotting demonstrated no difference in EGFR gene copy number between controls and ErbB2 transfectants (G. Huang, data not shown). This finding is consistent with the possibility that ErbB2-dependent heterodimerization of EGFR is responsible for the observed downregulatory defect.

DISCUSSION

Autocrine and paracrine growth loops have long been proposed as pathways for tumorigenesis, and in vitro support for this paradigm is strong [Reiss et al., 1991; van de Vijver et al., 1991]. An unresolved difficulty with this hypothesis concerns the efficient downregulation of receptor tyrosine kinases in response to ligand exposure—a negative feedback loop that might be expected to render constitutive overexpression of a ligand insufficient for tumorigenesis in otherwise normal tissues. We are unaware of any human cancer in which a growth factor gene is amplified, an event that would tend to confirm a clonally selectable phenotype. By contrast, amplification of the ErbB2 gene is one of the most characteristic genetic hallmarks of human malignant and precancerous neoplasms [Slamon et al., 1987]. Hence, considering the orphan status of ErbB2, it is plausible that this "receptor" exerts its tumorigenetic function through an indirect mechanism involving a hetero-oligomeric interplay with liganded growth factor receptors. Consistent with this possibility, downregulation of ErbB2 has been reported in response to heterologous growth factors [Oshima et al., 1995].

The present study shows that in vitro overexpression of ErbB2 impairs EGF-dependent downregulation EGFR. This finding suggests that human tumor cells could obtain a growth advantage from prolongation of EGFR expression, thereby enhancing cell sensitivity to the

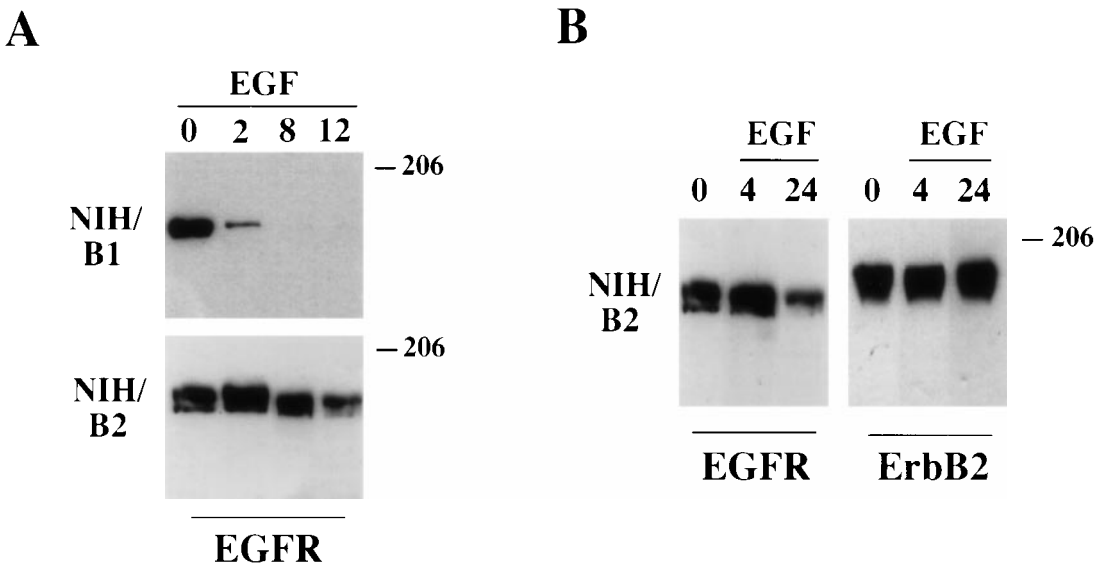

Fig. 4. Effect of ErbB2 expression on EGF-inducible epidermal growth factor receptor (EGFR) downregulation in NIH 3T3 cells. **A:** Time course of EGF-inducible EGFR downregulation in NIH 3T3 cells transfected with either human EGFR (NIH/B1, top) or ErbB2 (NIH/B2, bottom). The number of hours treatment with EGF (100 ng/ml) is indicated above each lane of the EGFR immunoblot. **B:** Relationship between EGF-inducible EGFR downregulation and ErbB2 expression levels in ErbB2 transfectants (NIH/B2). Cells were treated with EGF (100 ng/ml) for the duration in hours indicated above each lane, then subjected to lysis and immunoblotting using antibody to EGFR (left) or ErbB2 (right). These results were reproduced in two further experiments.

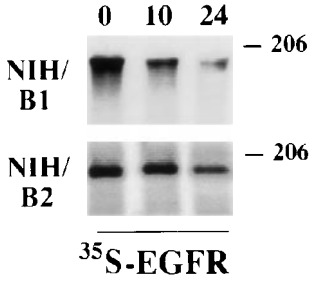

Fig. 5. EGFR turnover kinetics after metabolic labelling of epidermal growth factor receptor (EGFR)- (NIH/B1) and ErbB2-transfected (NIH/B2) 3T3 cells. Cells were labeled overnight with ^{35}S-methionine, treated with EGF (100 ng/ml) or diluent alone, then washed and re-fed with medium containing unlabeled methionine before lysis, immunoprecipitation, and electrophoresis. In the absence of a rodent-specific immunoprecipitating EGFR antibody, protein lysates were first denatured by boiling, then immunoprecipitated using an EGFR antibody (clone 13, Transduction Labs) and electrophoresed. The results represent one of two similar experiments.

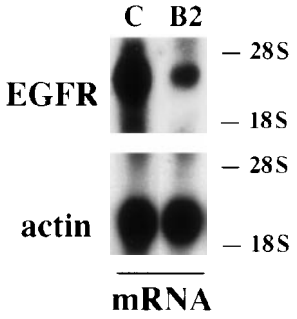

Fig. 6. Epidermal growth factor receptor (EGFR) gene expression in parental control (C; **lane 1**) and ErbB2-transfected NIH 3T3 cells (B2; **lane 2**) assessed by Northern blot analysis. RNA preparation and electrophoresis was carried out as described in Materials and Methods, and similar results were obtained in repeated experiments. Blots are shown for EGFR (top, showing the 5-kb transcript) and actin (bottom) genes; ribosomal RNA markers are shown at right.

mitogenic action of ambient EGFR ligands. Indeed, we have noted ligand-dependent heterodimerization of ErbB2 with non-type I receptor tyrosine kinases, which are also upregulated in stable ErbB2 transfectants (G. Huang, unpublished data), raising the possibility that overexpression of ErbB2 could represent a general mechanism for slowing the degradation of heterologous ligand-activated receptors and thereby prolonging growth factor signaling (Fig. 7).

The association of reduced EGFR mRNA with ErbB2 expression in 3T3 transfectants (Fig. 6) is reminiscent of the reported inhibition of EGFR gene expression in tumor cell lines constitutively secreting EGFR ligands [King and Sartorelli, 1989]. We recently reported that transforming growth factor-α (TGF-α) selectively fails to downregulate EGFR [Gulliford et al., 1997],

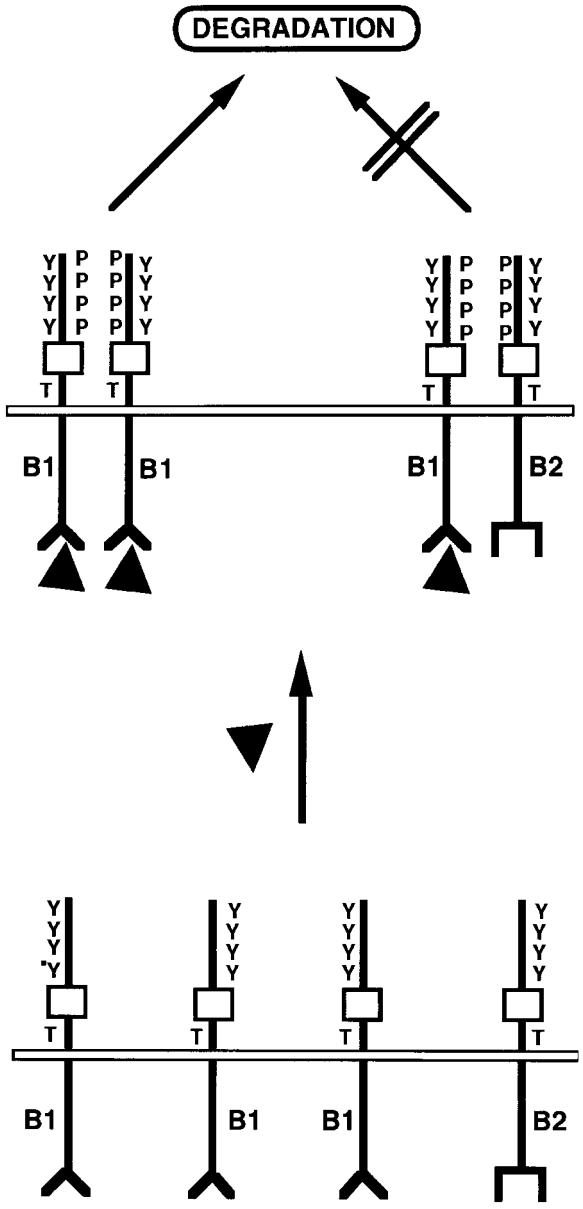

Fig. 7. Model of ErbB2-dependent impairment of epidermal growth factor receptor EGFR downregulation. After exposure to EGF (▼), EGFR (B1) homodimers and ErbB2 (B2) heterodimers are formed, leading to tyrosine phosphorylation and receptor internalization. Whereas the B1 homodimer is targeted for downregulation, the B2 heterodimer escapes degradation and can thus continue to signal following ligand treatment.

a finding since supported by intracellular trafficking studies [Lenferink et al., 1998], and have more recently linked this downregulatory impairment to the short duration of intracellular receptor ligation induced by this acid-labile ligand [Ouyang et al., 1998]. Hence, the present study suggests that ErbB2, like TGF-α, acts to prolong ligand-receptor signaling and that these two mechanisms may act in synergy to promote tumor growth [Muller et al., 1996]. Further work is needed to determine whether this ability of ErbB2 to act as an amplifier of ambient growth factor function is a more critical feature of ErbB2-dependent oncogenesis than signals originating from the ErbB2 catalytic domain itself.

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REFERENCES

Allred DC, Clark GM, Tandon AK, Molina R, Tormey DC, Osborne CK, Gilchrist KW, Mansour EG, Abeloff M, Eudey L, et al. 1992. HER-2/neu in node-negative breast cancer: prognostic significance of overexpression influenced by the presence of in situ carcinoma. J Clin Oncol 10:599–605.

Bargmann CI, Hung M, Weinberg RA. 1986. The *neu* oncogene encodes an epidermal growth factor receptor-related protein. Nature 319:226–230.

Baulida J, Kraus MH, Alimandi M, Di Fiore PP, Carpenter G. 1996. All erbB receptors other than the epidermal growth factor receptor are endocytosis impaired. J Biol Chem 271:5251–5257.

Chantry A. 1995. The kinase domain and membrane localization determine intracellular interactions between epidermal growth factor receptors. J Biol Chem 270:3068–3073.

Chen C, Okayama H. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 7:2745–2752.

Decker SJ, Alexander C, Habib T. 1992. Epidermal growth factor (EGF)-stimulated tyrosine phosphorylation and EGF receptor degradation in cells expressing EGF receptors truncated at residue 973. J Biol Chem 267:1104–1108.

Di Fiore PP, Pierce JH, Kraus KH, Segatto D, King CR, Aaronson SA. 1987. erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science 237:178–181.

Di Marco E, Pierce JH, Knicley CL, DiFiore PP. 1990. Transformation of NIH 3T3 cells by overexpression of the normal coding sequence of the rat neu gene. Mol Cell Biol 10:3247–3252.

Epstein RJ. 1995. Preferential detection of catalytically inactive c-erbB-2 by antibodies to unphosphorylated peptides mimicking receptor tyrosine autophosphorylation sites. Oncogene 11:315–323.

Epstein RJ, Druker BJ. 1990. Modulation of a M_r 175,000 c-neu receptor isoform in G8/DHFR cells by serum starvation. J Biol Chem 265:10746–10751.

Felder S, Miller K, Moehren G, Ullrich A, Schlessinger J, Hopkins CR. 1990. Kinase activity controls the sorting of epidermal growth factor receptor within the multivesicular body. Cell 61:623–634.

French AR, Sudlow GP, Wiley HS, Lauffenburger DA. 1994. Postendocytic trafficking of epidermal growth factor-receptor complexes is mediated through saturable and specific endosomal interactions. J Biol Chem 269:15749–15755.

Galcheva-Gargova Z, Theroux SJ, Davis RJ. 1995. The epidermal growth factor receptor is covalently linked to ubiquitin. Oncogene 11:2649–2655.

Glenney JR, Chen WS, Lazar CS, Walton GM, Zokas LM, Rosenfeld MG, Gill GN. 1988. Ligand-induced endocytosis of the EGF receptor is blocked by mutational inactivation and by microinjection of antiphosphotyrosine antibodies. Cell 52:675–684.

Gulliford T, Huang GC, Ouyang X, Epstein RJ. 1997. Reduced ability of transforming growth factor-alpha to induce hetero-oligomerization and downregulation of EGFR suggests a mechanism of oncogenic synergy with ErbB2. Oncogene 15:2219–2223.

Helin K, Beguinot L. 1991. Internalization and down regulation of the human epidermal growth factor receptor are regulated by the carboxyl-terminal tyrosines. J Biol Chem 266:8363–8368.

Hoffman P, Carlin C. 1994. Adenovirus E3 protein causes constitutively internalized epidermal growth factor receptors to accumulate in a prelysosomal compartment, resulting in enhanced degradation. Mol Cell Biol 14:3695–3706.

Hopkins CR. 1992. Selective membrane protein trafficking: vectorial flow and filter. Trends Biochem Sci 17:27–32.

Huang GC, Ouyang X, Epstein RJ. 1998. Proxy activation of ErbB2 by heterologous ligands suggests a heterotetrameric mechanism of receptor tyrosine kinase interaction. Biochem J 331:113–119.

Huang HS, Nagane M, Klingbeil CK. 1997. The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. J Biol Chem 272:2927–2935.

Kawamoto T, Sato JD, Le A, Polikoff J, Sato GH, Mendelsohn J. 1983. Growth stimulation of A431 cells by epidermal growth factor: identification of high-affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. Proc Natl Acad Sci USA 80:1337–1341.

King CR, Borrello I, Bellot F, Comoglio P, Schlessinger J. 1988. EGF binding to its receptor triggers a rapid tyrosine phosphorylation of the erbB-2 protein in the mammary tumor cell line SK-BR-3. EMBO J 7:1647–1651.

King I, Sartorelli AC. 1989. Epidermal growth factor receptor gene expression, protein kinase activity, and terminal differentiation of human malignant epidermal cells. Cancer Res 49:5677–5681.

Kokai Y, Myers JN, Wada T, Brown VI, LeVea CM, Davis JG, Dobashi K, Greene MI. 1989. Synergistic interaction of p185/c-neu and the EGF receptor leads to transformation of rodent fibroblasts. Cell 58:287–292.

Kornilova E, Sorkina T, Beguinot L, Sorkin A. 1996. Lysosomal targeting of epidermal growth factor receptors via a kinase-dependent pathway is mediated by the receptor carboxyterminal residues 1022–1123. J Biol Chem 271: 30340–30346.

Kurten RC, Cadena DL, Gill GN. 1996. Enhanced degradation of EGF receptors by a sorting nexin, SNX1. Science 272:1008–1010.

Kwatra MM, Bigner DD, Cohn JA. 1992. The ligand binding domain of the epidermal growth factor receptor is not required for receptor dimerization. Biochim Biophys Acta 1134:178–181.

Lamaze C, Schmid SL. 1995. Recruitment of epidermal growth factor receptors into coated pits requires their activated tyrosine kinase. J Cell Biol 129:47–54.

Lemoine NR, Staddon S, Dickson C, Barnes DM, Gullick WJ. 1990. Absence of activating transmembrane mutations in the c-erbB-2 proto-oncogene in human breast cancer. Oncogene 5:237–239.

Lenferink AE, Pinkas-Kramarski R, van de Poll ML, van Vugt MJ, Klapper LN, Tzahar E, Waterman H, Sela M, van Zoelen EJ, Yarden Y. 1998. Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. EMBO J 17:3385–3397.

Mimnaugh EG, Chavany C, Neckers L. 1996. Polyubiquitinylation and proteasomal degradation of the p185$^{c\text{-}crbB\text{-}2}$ receptor protein-tyrosine kinase induced by geldenamycin. J Biol Chem 271:22796–22801.

Mori S, Tanaka K, Omura S, Saito Y. 1995. Degradation process of ligand-stimulated platelet-derived growth factor β-receptor involves ubiquitin-proteasome proteolytic pathway. J Biol Chem 270:29447–29452.

Muller WJ, Arteaga CL, Muthuswamy SK, et al. 1996. Synergistic interaction of the neu proto-oncogene product and transforming growth factor alpha in the mammary epithelium of transgenic mice. Mol Cell Biol 16:5726–5736.

Nesterov A, Wiley HS, Gill GN. 1995. Ligand-induced endocytosis of epidermal growth factor receptors that are defective in binding adaptor proteins. Proc Natl Acad Sci USA 92:8719–8723.

Opresko LK, Chang C, Will BH, Burke PM, Gill GN, Wiley HS. 1995. Endocytosis and lysosomal targeting of epidermal growth factor receptors are mediated by distinct sequences independent of the tyrosine kinase domain. J Biol Chem 270:4325–4333.

Oshima M, Weiss L, Dougall WC, Greene MI, Guroff G. 1995. Down-regulation of c-neu receptors by nerve growth factor in PC12 cells. J Neurochem 65:427–433.

Ouyang X, Gulliford T, Chantry A, Epstein RJ. 1996. Human cancer cells exhibit protein kinase C-dependent c-erbB-2 transmodulation which correlate with phosphatase sensitivity and kinase activity. J Biol Chem 271: 21786–21792.

Ouyang X, Gulliford T, Huang GC, Epstein RJ. 1999. Transforming growth factor-alpha short-circuits downregulation of the epidermal growth factor receptor. J Cell Physiol (in press)

Pandit SD, Donis-Keller H, Iwamoto T, Tomich JM, Pike LJ. 1996. The multiple endocrine neoplasia type 2B point mutation alters long-term regulation and enhances the transforming capacity of the epidermal growth factor receptor. J Biol Chem 271:5850–5858.

Reiss M, Stash EB, Vellucci VF, Zhou Z. 1991. Activation of the autocrine transforming growth factor alpha pathway in human squamous carcinoma cells. Cancer Res 51:6254–6262.

Robinson MS. 1987. 100-kD coated vesicle proteins: molecular heterogeneity and intracellular distribution studied with monoclonal antibodies. J Cell Biol 104:887–895.

Skehan P, Storeng R, Scudeiro D, et al. 1990. New colorimetric cytotoxicity assay for anticancer drug screening. J Natl Cancer Inst 82:1107–1112.

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. 1987. Human breast cancer: correlation of

relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177–182.

Sorkin A, Carpenter G. 1993. Interaction of activated EGF receptors with coated pit adaptins. Science 261:612–615.

Sorkin A, Mazzotti M, Sorkina T, Scotto L, Beguinot L. 1996. Epidermal growth factor receptor interaction with clathrin adaptors is mediated by the Tyr^{974}-containing internalization motif. J Biol Chem 271:13377–13384.

Sorkin A, McKinsey T, Shih W, Kirchhausen T, Carpenter G. 1995. Stoichiometric interaction of the epidermal growth factor receptor with the clathrin-associated protein complex AP-2. J Biol Chem 270:619–625.

Stoscheck CM, Carpenter G. 1984. Down regulation of epidermal growth factor receptors: direct demonstration of receptor degradation in human fibroblasts. J Cell Biol 98:1048–1053.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Ullrich A, Schlessinger J. 1990. Signal transduction by receptors with tyrosine kinase activity. Cell 61:203–212.

van de Vijver MJ, Kumar R, Mendelsohn J. 1991. Ligand-induced activation of A431 cell epidermal growth factor receptors occurs primarily by an autocrine pathway that acts upon receptors on the surface rather than intracellularly. J Biol Chem 266:7503–7508.

Warren RA, Green FA, Enns CA. 1997. Saturation of the endocytic pathway for the transferrin receptor does not affect the endocytosis of the epidermal growth factor receptor. J Biol Chem 272:2116–2121.

Wells A, Welsh J, Lazar C, Wiley H, Gill G, Rosenfeld M. 1990. Ligand-induced transformation by a noninternalizing epidermal growth factor receptor. Science 247:962–964.