MOLECULAR AND CELLULAR BIOLOGY, June 1990, p. 2749–2756
0270-7306/90/062749-08$02.00/0

Vol. 10, No. 6

The Carboxy-Terminal Domains of *erbB-2* and Epidermal Growth Factor Receptor Exert Different Regulatory Effects on Intrinsic Receptor Tyrosine Kinase Function and Transforming Activity

PIER PAOLO DI FIORE, ORESTE SEGATTO, FULVIO LONARDO, FRANCESCA FAZIOLI, JACALYN H. PIERCE, AND STUART A. AARONSON*

Laboratory of Cellular and Molecular Biology, National Cancer Institute, Building 37, Room 1E24, Bethesda, Maryland 20892

Received 5 September 1989/Accepted 8 March 1990

The *erbB-2* gene product, gp185^{erbB-2}, displays a potent transforming effect when overexpressed in NIH 3T3 cells. In addition, it possesses constitutively high levels of tyrosine kinase activity in the absence of exogenously added ligand. In this study, we demonstrate that its carboxy-terminal domain exerts an enhancing effect on *erbB-2* kinase and transforming activities. A premature termination mutant of the *erbB-2* protein, lacking the entire carboxy-terminal domain (*erbB-2*$^{\Delta1050}$), showed a 40-fold reduction in transforming ability and a lowered in vivo kinase activity for intracellular substrates. When the carboxy-terminal domain of *erbB-2* was substituted for its analogous region in the epidermal growth factor receptor (EGFR) (EGFR/*erbB-2*^{COOH} chimera), it conferred *erbB-2*-like properties to the EGFR, including transforming ability in the absence of epidermal growth factor, elevated constitutive autokinase activity in vivo and in vitro, and constitutive ability to phosphorylate phospholipase C-γ. Conversely, a chimeric *erbB-2* molecule bearing an EGFR carboxy-terminal domain (*erbB-2*/EGFR^{COOH} chimera) showed reduced transforming and kinase activity with respect to the wild-type *erbB-2* and was only slightly more efficient than the *erbB-2*$^{\Delta1050}$ mutant. Thus, we conclude that the carboxy-terminal domains of *erbB-2* and EGFR exert different regulatory effects on receptor kinase function and biological activity. The up regulation of gp185^{erbB-2} enzymatic activity exerted by its carboxy-terminal domain can explain, at least in part, its constitutive level of kinase activity.

Signal transduction mediated by growth factor interaction with specific cell surface receptors is of critical importance in the regulation of normal cell growth and differentiation (14, 21). Many growth factor receptors belong to a family of evolutionarily conserved proteins that possess intrinsic tyrosine kinase activity (16). These membrane-spanning proteins possess an extracellular ligand-binding domain joined to an intracellular domain through a short transmembrane region composed of hydrophobic amino acids (12, 30, 41, 50, 51). The intracellular portion is thought to be responsible for transducing the mitogenic signal by phosphorylating specific intracellular substrates (16, 29, 32, 48). Moreover, genetic manipulations which destroy the intrinsic tyrosine kinase activity of these molecules also abolish their biological effects (5, 10, 17–19, 33).

The epidermal growth factor (EGF) receptor (EGFR) and the *erbB-2* receptor-like protein belong to a subfamily of tyrosine kinase growth factor receptors which share extensive structural and sequence homologies. Several lines of evidence, however, indicate that the enzymatic activities of EGFR and *erbB-2* might be differently regulated in vivo. First, mutations known to activate the *erbB-2* tyrosine kinase (2–4, 39) do not affect this activity for the EGFR (22). Moreover, the *erbB-2* product, unlike the EGFR, possesses high levels of in vivo tyrosine phosphorylation in the absence of exogenously added ligand (45; O. Segatto, J. H. Pierce, D. P. Bottaro, and P. P. Di Fiore, New Biologist, in press). This has been reproducibly observed in a variety of cell systems, including naturally occurring adenocarcinomas (11, 24, 26, 40, 42, 43, 53) as well as in vitro-generated model systems in which the *erbB-2* protein has been overexpressed in fibroblasts (45; Segatto et al., in press), hematopoietic cells (P. P. Di Fiore, O. Segatto, S. A. Aaronson, and J. P. Pierce, Science, in press), and epithelial cells (our published observations). While it is possible that such diverse cell types all constitutively synthesize its ligand, an alternative possibility is that *erbB-2* may encode an atypical receptor-like molecule, whose activity is regulated by its levels of expression and the availability of a critical intracellular substrate(s).

This study was undertaken to study *erbB-2* function with regard to its apparent constitutive activation. To investigate the structural basis for this phenomenon, we focused on the *erbB-2* carboxy-terminal domain because of the high primary structural divergence between EGFR and *erbB-2* products in that region (6, 46) and because a number of studies have indicated a heterogeneous regulatory role for this domain in other growth factor receptors and receptor-like molecules (10, 15, 28, 31, 47).

MATERIALS AND METHODS

Cell culture and transfection assays. The continuous mouse NIH 3T3 (1) and NR6 (36) cell lines have been previously described; they were cultivated in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% calf serum (GIBCO). DNA transfection of NIH 3T3 and NR6 cells was performed by the calcium phosphate precipitation technique (13), as modified by Wigler et al. (49). Transformed foci were scored at 3 weeks. Where indicated, EGF (20 ng/ml) was added at day 14 and foci were scored at day 21. Transforming efficiency was calculated in focus-forming units (FFU) per picomole of added DNA after correction for the relative molecular weight of the respective plasmid and normalization for the efficiency of colony formation in par-

* Corresponding author.

allel dishes subjected to selection in mycophenolic acid-containing medium. Cells expressing the *Ecogpt* gene were selected for their ability to form colonies in the presence of a mycophenolic acid-containing medium (34).

Engineering of eucaryotic expression vectors. All of the molecular constructs utilized were derived from either the LTR-2/*erbB-2* (8) or LTR-2/EGFR (7) expression vectors previously described. The engineering of the *erbB-2*$^{\Delta 1076}$ carboxy-terminal truncated mutant has been previously reported (O. Segatto et al., in press). The *erbB-2*$^{\Delta 1050}$ mutant was engineered by site-directed mutagenesis, by the method of Kunkel (27), by mutagenizing the TCA (Ser) codon at position 1050 of the *erbB-2* open reading frame (6) to TAA.

The LTR-EGFR/*erbB-2*^{COOH} and LTR-*erbB-2*/EGFR^{COOH} expression vectors were engineered starting from the LTR-2/*erbB-2* (8) and LTR-2/EGFR (7) plasmids, as previously described (Di Fiore et al., in press). Briefly, a conserved *Bcl*I site, at positions 3009 and 3021 of the original *erbB-2* (6) and EGFR (46) sequences, respectively, and a unique *Cla*I site contained in the Moloney murine leukemia virus sequence following the stop codons of both genes in the LTR vectors were used for the recombination. Both LTR-2/EGFR and LTR/*erbB-2* were digested with *Bcl*I and *Cla*I. A 2.1-kilobase *Bcl*I-*Cla*I fragment from LTR-2/*erbB-2* containing the 3′ end of *erbB-2* cDNA was ligated to the 11.2-kilobase *Bcl*I-*Cla*I fragment of the LTR/EGFR containing the EGFR cDNA from which the 3′ terminus was deleted. This yielded the LTR-EGFR/*erbB-2*^{COOH} expression vector. The reciprocal recombination yielded the LTR-*erbB-2*/EGFR^{COOH} expression vector. Because of the presence of a second *Bcl*I site at position 3078 in the EGFR cDNA (46), an oligonucleotide was used to restore 19 codons of the EGFR encompassed between the two *Bcl*I sites. All the constructions described were sequenced in both strands of the regions which underwent genetic manipulation to verify that the predicted structures were achieved after the recombination procedures.

Protein analysis. For Western blot (immunoblot) analysis, cells were grown until they were 90% confluent and then lysed in a lysis buffer containing 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, 0.01 M sodium phosphate (pH 7.4), 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium PP_i, and 400 μM sodium orthovanadate. Lysates were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto nitrocellulose, as previously described (8, 39). Immunodetection was performed with the indicated antibodies coupled to ^{125}I-labeled protein A, as described previously (8, 39). The immune sera used were as follows: M1 serum, generated against an *erbB-2* peptide encompassing residues 866 through 880 of the predicted *erbB-2* sequence; M6 serum, directed against the *erbB-2* peptide (residues 1218 through 1231); M7 serum, directed against the *erbB-2* peptide (residues 1240 through 1255); E5 serum, directed against the EGFR peptide (residues 985 through 996); E7 serum, directed against the EGFR peptide (residues 1172 through 1186); and anti-F serum, directed against the protein kinase C modulation domain of the EGFR (kindly provided by J. Schlessinger). The M1 and M6 antibodies recognized equally well the phosphorylated and dephosphorylated *erbB-2* molecules. In fact, *erbB-2* from total cell lysates done in the absence of phosphatase inhibitors was shown to be completely depleted of phosphotyrosine (PTyr). In this condition, *erbB-2* was recognized with the same efficiency as *erbB-2* in lysates done in the presence of phosphatase inhibitors (results not shown). The following three different anti-PTyr antibodies were utilized: a polyclonal serum prepared against a conjugate of keyhole limpet hemocyanine and phosphotyramine as described by Pang et al. (35), the same polyclonal serum after affinity purification on agarose-coupled phosphotyramine (35), and a commercially available anti-PTyr monoclonal antibody (PY20; ICN Pharmaceuticals, Inc.). The anti-PTyr immunostainings shown were obtained with the affinity-purified polyclonal serum. However, three antibodies gave similar results. The specificity of immunodetection for the peptide antisera was controlled by performing parallel staining of identical blots with antibodies preabsorbed with the specific peptides (2 mg/ml). In the case of the anti-PTyr antibody, specificity was controlled by preabsorption of the antibody with either PTyr, phosphoserine, or phosphothreonine.

The in vivo autophosphorylation assay for EGFR and EGFR/*erbB-2*^{COOH} was performed by a treatment of cell lines with EGF (100 ng/ml) for 5 min at 37°C followed by lysis and immunoblotting analysis as described previously (9).

In vitro autophosphorylation assays were performed on cell membrane preparations. For membrane fractionation, cells were lysed in hypotonic buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.4, with the aid of a Dounce homogenizer, pestle A. The nuclear fraction was removed by centrifugation at 600 × *g*. Subsequently, membranes were pelleted at 100,000 × *g* for 45 min and suspended in a buffer containing 50 mM HEPES (pH 7.4), 0.1 M NaCl, 1 mM phenylmethylsulfonyl fluoride, and no phosphatase inhibitors. Where indicated, treatment with EGF (300 ng/ml) was performed for 5 min at 25°C. The kinase assay was carried out in the presence of 60 μM cold ATP and 10 μM $MgCl_2$ for the indicated lengths of time at either 4 or 25°C. Where indicated, 250 mM ammonium sulfate was added to the reaction mix. The reaction was blocked with the addition of Laemmli sample buffer, and samples were analyzed by SDS-PAGE followed by Western blot, as described previously (8, 39).

Phospholipase C-γ (PLC-γ) in vivo tyrosine phosphorylation was detected by sequential immunoprecipitation with anti-PTyr followed by immunoblot with a mixture of six monoclonal antibodies directed against PLC-γ (kindly provided by S. G. Rhee).

RESULTS

The carboxy-terminal domain participates in the regulation of *erbB-2* biological activity. We have demonstrated that tyrosine phosphorylation of the *erbB-2* gene product, which occurs in the carboxy-terminal (COOH) domain, exerts a modest regulatory effect on its biological activity. Accordingly, a COOH-deleted mutant (*erbB-2*$^{\Delta 1076}$) which showed a dramatic reduction in PTyr content displayed only a fivefold reduction in transforming ability (Segatto et al., in press). To more precisely determine the role of the COOH domain in *erbB-2* function, we engineered a COOH-deleted mutant at position 1050 of the *erbB-2* predicted protein sequence (6). This mutant, designated *erbB-2*$^{\Delta 1050}$, encodes a protein lacking the great majority of the COOH domain of *erbB-2*, as defined by the absence of detectable homology with the structurally related EGFR (46). The *erbB-2*$^{\Delta 1076}$ mutant, bearing a deletion of 179 amino acids, showed around 20% of the wild-type (wt) *erbB-2*-transforming efficiency, whereas the *erbB-2*$^{\Delta 1050}$ mutant, bearing a further 26-amino-acid deletion, showed only 2.5% of the activity observed for the full-length *erbB-2* molecule (Fig. 1A).

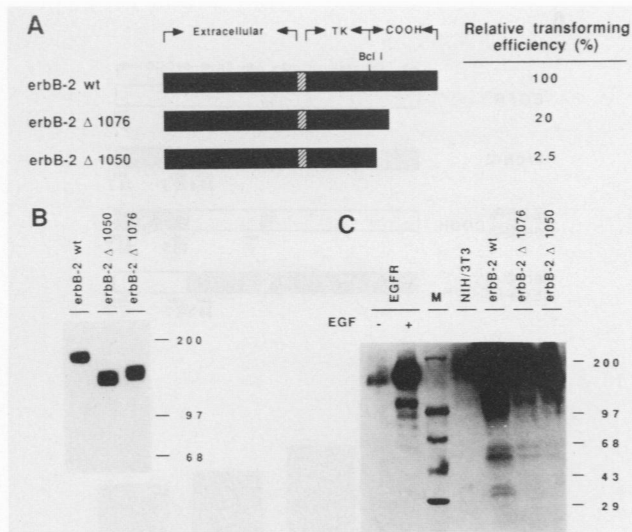

FIG. 1. Transforming activities and biochemical analysis of *erbB-2* COOH-deleted mutants transfected in NIH 3T3 fibroblasts. (A) Transforming activity. The LTR/*erbB-2* (8), LTR/*erbB-2*$^{\Delta 1076}$ and LTR/*erbB-2*$^{\Delta 1050}$ expression vectors were transfected into NIH 3T3 cells at 10-fold serial dilutions by the calcium phosphate precipitation technique (13), with 40 μg of calf thymus DNA as a carrier. Assays were scored at 21 days as previously described (7, 8). Transforming efficiency was calculated in FFU per picomole of added DNA, after correction for the relative molecular weight of the respective plasmid and normalization for the efficiency of colony formation in parallel dishes treated with mycophenolic acid-containing medium (34). Typically, we obtained a transforming efficiency of around 10^4 FFU/pmol and 10^4 mycophenolic acid-resistant colonies per pmol when linearized LTR/*erbB-2* was used. The relative transforming efficiency was calculated on the basis of the following formula: [(FFU/pmol of mutants)/(FFU/pmol of wt *erbB-2*)] × 100. Data represent averages from at least four independent experiments performed in duplicate. Standard error was less than 10% of the mean. TK, Tyrosine kinase. Symbols: ■, *erbB-2* sequence; ▨, transmembrane sequence. (B) Comparison of steady-state expression of wt and mutant *erbB-2* proteins in NIH 3T3 transfectants. Mass cell populations were obtained by selection in a medium containing mycophenolic acid. Total cellular proteins (50 μg) from NIH 3T3 cells transfected with the indicated DNAs were separated by SDS-PAGE and subjected to immunoblot analysis with the M1 antibody. Molecular mass markers are shown in kilodaltons. Data are typical and representative of three independent experiments. (C) Tyrosine phosphorylation of cellular proteins in NIH 3T3 cells transfected with wt *erbB-2*, COOH-deleted *erbB-2* mutants, or EGFR. Mass cell populations from NIH 3T3 cells transfected with the indicated DNAs were obtained by selection in a medium containing mycophenolic acid. Total cell lysates (200 μg) were fractionated by SDS-PAGE and subjected to immunoblot analysis. Immunodetection was carried out with an affinity-purified anti-PTyr antibody, as described in Materials and Methods. The autoradiogram is overexposed to reveal low-abundance tyrosine-phosphorylated proteins. Molecular mass markers (M) are shown in kilodaltons. Results are typical and representative of at least three independent experiments. NIH-EGFR cells were treated with (+) or without (−) EGF, as described in Materials and Methods.

To analyze expression of the COOH-terminal mutants, we established stable mass populations of transfected cell lines by selection with mycophenolic acid. Total cell lysates were subjected to Western blot analysis by using the M1 antipeptide serum which recognizes an epitope (residues 866 through 880) present in the wt *erbB-2* protein and its COOH mutants. This antibody also recognized with equal efficiency tyrosine-phosphorylated or -dephosphorylated *erbB-2* (see Materials and Methods). All mutant proteins were expressed at levels similar to those detected in NIH 3T3 cells transfected with the wt *erbB-2* expression vector (Fig. 1B). The COOH-deleted proteins also exhibited faster migration in SDS-PAGE than the wt gp185$^{erbB\text{-}2}$, consistent with the extent of their respective deletions (Fig. 1B). Moreover, antipeptide sera (M6 and M7 antibodies; see Materials and Methods) directed against determinants not retained in the mutant proteins failed to recognize them but readily detected the wt gp185$^{erbB\text{-}2}$ (data not shown). Taken together, these results indicated that the reduced biological activity of the *erbB-2* mutants was not due to a decrease in their level of expression but rather to their altered structure. Thus, our results indicated that the COOH domain has a positive regulatory role in *erbB-2* biological activity.

To investigate the molecular basis of the regulatory role of the *erbB-2* COOH domain, we analyzed the pattern of tyrosine-phosphorylated proteins in cells overexpressing wt gp185$^{erbB\text{-}2}$ as well as COOH-deleted proteins. Very similar patterns of PTyr-containing proteins were detected in NIH 3T3 cells transformed by wt *erbB-2*, *erbB-2*$^{\Delta 1076}$, or *erbB-2*$^{\Delta 1050}$ (Fig. 1C). However, the mutants induced tyrosine phosphorylation of intracellular proteins with reduced efficiency, compared with wt gp185$^{erbB\text{-}2}$ (Fig. 1C). None of these putative substrates were identifiable in control NIH 3T3 cells. In addition, none of the putative substrate was recognized by the M1 antibody, thus indicating that they did not represent PTyr containing COOH-terminal degradation products of *erbB-2*. These findings suggested that COOH truncation led to reduced kinase activity in vivo or to lower substrate affinity. Of note, the observed pattern of tyrosine-phosphorylated proteins in *erbB-2* transformants displayed qualitative and quantitative differences when compared with the pattern detected in NIH 3T3 transfectants overexpressing the EGFR (7) and stimulated by EGF (Fig. 1B). This observation supports the concept that *erbB-2* and EGFR mediate their mitogenic effects through different or at least partially different signalling pathways (7, 8).

The carboxy-terminal domains of *erbB-2* and EGFR molecules differentially regulate their biological activities. To investigate the respective roles of their COOH domains, in determining their biological activity, we engineered chimeric molecules between *erbB-2* and EGFR by reciprocally switching their COOH domains. The transforming ability of these chimeras was then assessed by transfection of NIH 3T3 fibroblasts. Each chimera was found to differ from both of the parental molecules (Fig. 2). The EGFR/*erbB-2*^{COOH} chimera showed a basal transforming efficiency of 6.7×10^1 FFU/pmol in the absence of EGF stimulation. This was significantly above the undetectable level of transforming activity of the parental EGFR cDNA (Fig. 2). Both molecules were sensitive to EGF stimulation, which significantly enhanced their transforming ability. Conversely, the COOH domain of the EGFR exerted a negative effect on *erbB-2* biological activity. The *erbB-2*/EGFR^{COOH} chimera displayed around 20-fold lower transforming potency with respect to the parental *erbB-2* molecule (Fig. 2).

To ensure that these results were not due to different levels of expression of each protein, we used a panel of antipeptide sera directed against epitopes localized either to the tyrosine kinase or COOH domains of EGFR and *erbB-2*. An outline of the strategy employed is depicted in Fig. 3A. The relative levels of *erbB-2*, EGFR, *erbB-2*/EGFR^{COOH}, and EGFR/*erbB-2*^{COOH} proteins, detected by Western blot, were calculated on the basis of densitometric scans and

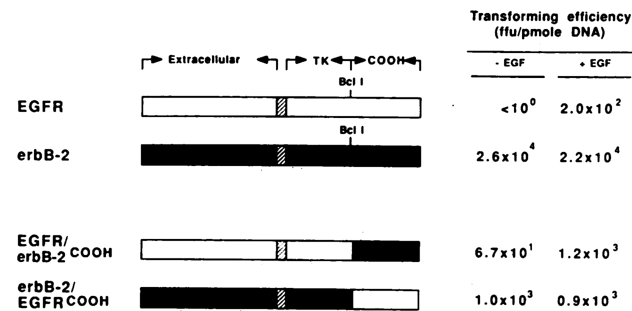

FIG. 2. Transforming activities of COOH chimeric molecules between EGFR and *erbB-2* in NIH 3T3 cells. Transfection was performed by the calcium phosphate precipitation technique (13) with 40 μg of calf thymus DNA as a carrier and 10-fold serial dilutions of linearized plasmid DNA. Assays were scored at 21 days. Where indicated, EGF (20 ng/ml) was added at day 14 and assays were scored at day 21. Transforming efficiency was calculated in FFU per picomole of added DNA, as indicated in the legend to Fig. 1. Data represent the average of three independent experiments performed in duplicate. Standard error was less than 10% of the mean. TK, Tyrosine kinase. Symbols: □, EGFR sequence; ■, *erbB-2* sequence; ▨, transmembrane sequence.

titration analysis. No major differences in the levels of the expressed proteins were detected (Fig. 3B).

***erbB-2* and EGFR COOH domains exert differential regulatory effects on tyrosine kinase activity.** The level of in vivo tyrosine phosphorylation has been shown to be a reliable marker of receptor activation both for EGFR and *erbB-2* (4, 5, 9, 18, 19, 33, 38, 39, 45). To examine the possibility that their COOH domains differentially affected the intrinsic enzymatic activities of *erbB-2* and EGFR molecules, we analyzed in vivo tyrosine phosphorylation of the chimeric proteins in comparison with the parental molecules. EGFR and EGFR/*erbB-2*^{COOH} proteins, analyzed in overexpressing cell lines, were found to possess different biochemical properties (Fig. 4A and B). As previously reported (9, 38), EGFRs showed little if any tyrosine phosphorylation in cells cultivated in the absence of EGF but demonstrated rapid tyrosine phosphorylation in response to the addition of EGF. Conversely, the EGFR/*erbB-2*^{COOH} chimera showed a detectable basal level of tyrosine phosphorylation, which was further augmented in response to EGF (Fig. 4A and B). The same results were reproducibly observed in NR6 transfectants as well (Fig. 4A). Since this latter cell line is devoid of EGFR (36), these findings excluded the possibility that the level of unstimulated tyrosine phosphorylation of the EGFR/*erbB-2*^{COOH} chimera was due to intracellular cross talk between this molecule and endogenous EGFR (23, 25, 44).

The introduction of the EGFR COOH domain to the *erbB-2* molecule was associated with a decrease in the level of in vivo tyrosine phosphorylation, compared with the *erbB-2* parental molecule. The *erbB-2*/EGFR^{COOH} chimera displayed around 50% lower levels of tyrosine phosphorylation than the wild-type *erbB-2* molecule (Fig. 4B). All of these results were consistent with the concept that the EGFR COOH domain down modulated the intrinsic tyrosine kinase activity of either EGFR or *erbB-2* proteins, whereas this domain of *erbB-2* exerted up regulation of the tyrosine kinase activity of both molecules.

These in vivo findings were paralleled by results obtained in an in vitro autophosphorylation assay of EGFR and EGFR/*erbB-2*^{COOH} proteins. The EGFR/*erbB-2*^{COOH} chimeric protein displayed a higher basal level of autophosphorylation than EGFR in a series of in vitro assays performed in different temperatures, incubation times, and ionic strengths of buffer (Fig. 5). Both molecules also responded to the addition of the ligand with increased autocatalytic activity. These results further demonstrate that the carboxy-terminal domains differentially regulate the catalytic activities of EGFR and *erbB-2* in a manner which significantly influences their respective biological activities.

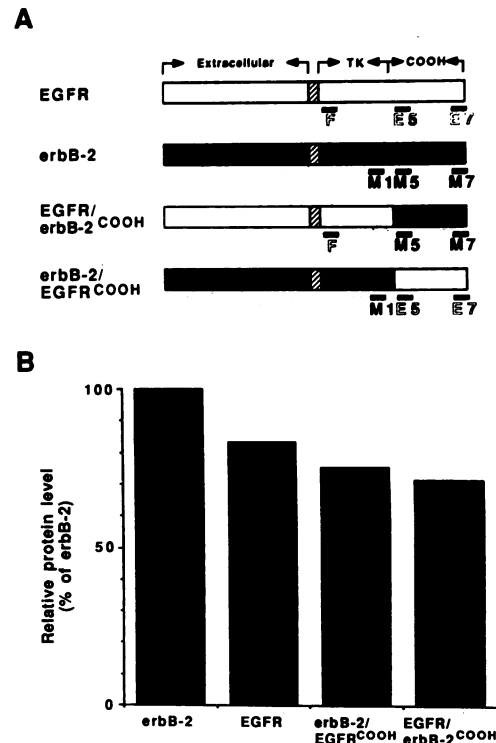

FIG. 3. Comparison of steady-state expression of wt EGFR, wt *erbB-2*, and COOH chimeras expressed in NIH 3T3 cells. Mass cell populations from NIH 3T3 cells transfected with the indicated DNAs were obtained by selection in a medium containing mycophenolic acid. Total cell extracts were then analyzed by immunoblot for the specific protein content. (A) Strategy of detection. Since no direct comparison among the indicated proteins was possible, given their different primary structures, we used a panel of antipeptide sera (F, E5, E7, M1, M5, and M7 antibodies described in Materials and Methods). The rationale of the approach was based on the fact that each antibody recognized a parental and a chimeric molecule. Moreover, each chimeric molecule was recognized by antibodies which also detected either one of the parental molecules. Total cell lysates at serial dilutions were analyzed by immunoblot with each of the indicated antibodies, and autoradiograms were subjected to densitometry. By matching the quantitative measurements obtained by densitometric scans and titration analysis, the relative protein levels were then calculated for each molecule and expressed as the percentage of the *erbB-2* level. TK, Tyrosine kinase. Symbols: □, EGFR sequence; ■, *erbB-2* sequence; ▨, transmembrane sequence. (B) Relative protein levels. Total cell lysates at twofold serial dilutions (50, 25, 12, and 6 μg), from the indicated cell lines, were fractionated by SDS-PAGE and transferred to nitrocellulose. Lysates were obtained in the absence of phosphatase inhibitors. Under these conditions, the receptor proteins were depleted of PTyr, as demonstrated by immunoblot with anti-PTyr antibodies (results not shown). Thus, the reactivity of the antipeptide antibodies could not be affected by tyrosine phosphorylation of the recognized epitope. Six identical blots were then immunostained with the six antipeptide antibodies indicated in panel A. Relative protein levels were calculated as described above. Results are typical and representative of three independent determinations.

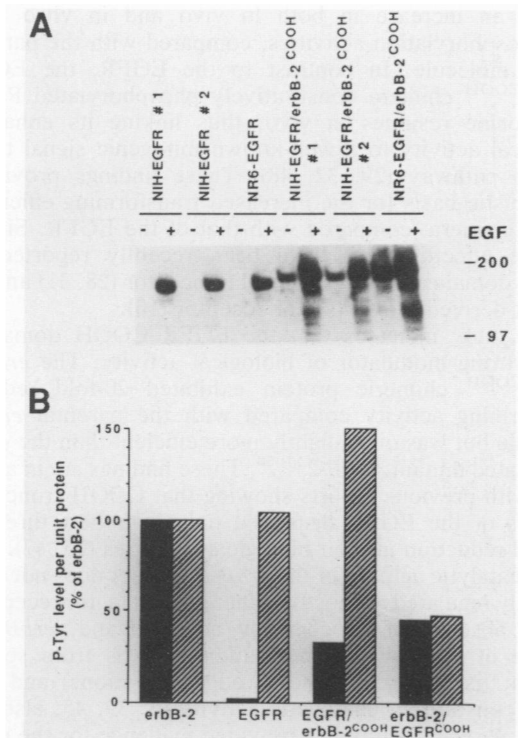

FIG. 4. In vivo tyrosine phosphorylation of EGFR, *erbB-2*, and COOH chimeras expressed in NIH 3T3 or NR6 cells. (A) Total cell lysates (50 μg) from the indicated cell lines were fractionated by SDS-PAGE and subjected to Western blot analysis. Immunodetection was carried out with the affinity-purified anti-PTyr antibody described in Materials and Methods. Where indicated (− or + EGF), EGF treatment (100 ng/ml) of intact cells was performed for 5 min at 37°C. Molecular mass markers are indicated in kilodaltons. (B) Equal amounts of specific receptor proteins were fractionated by SDS-PAGE. The amount of total cell lysates from transfected NIH 3T3 cells to be loaded on the gel to obtain equal quantities of specific receptor proteins was calculated on the basis of the values shown in Fig. 3B. However, in this experiment, lysates were done in the presence of phosphatase inhibitors. Proteins were transferred to nitrocellulose and immunodetected with the affinity-purified anti-PTyr antibody described in Materials and Methods. Treatment with EGF (100 ng/ml) was carried out at 37°C for 5 min, when indicated. Symbols: ■, no EGF treatment; ▨, EGF treatment. Quantitative measurements were obtained by densitometric scans and titration analysis, and the PTyr content of each molecule was calculated in arbitrary units and expressed as the percentage of the *erbB-2* PTyr level. Data are typical and representative of three independent determinations.

Increased in vivo tyrosine phosphorylation of PLC-γ by EGFR/*erbB-2*^{COOH} in comparison with EGFR. If the increased catalytic activity of the EGFR/*erbB-2*^{COOH} chimera resulted in a chronic activation of the EGF-responsive mitogenic pathway, one should expect evidence of constitutive tyrosine phosphorylation of intracellular substrates, which are normally phosphorylated by EGFR only upon EGF triggering. One such substrate, PLC-γ, has recently been identified (29, 32, 48). Thus, we investigated the state of PLC-γ tyrosine phosphorylation in EGFR and EGFR/*erbB-2*^{COOH} transfectants in the presence or absence of EGF stimulation. For these studies, two mass cell populations of NIH 3T3-EGFR and NIH 3T3-EGFR/*erbB-2*^{COOH} expressing comparable number of receptors were selected. ^{125}I-labeled EGF-binding experiments revealed that these two transfectants expressed 1.5×10^6 EGFR and 1.3×10^6

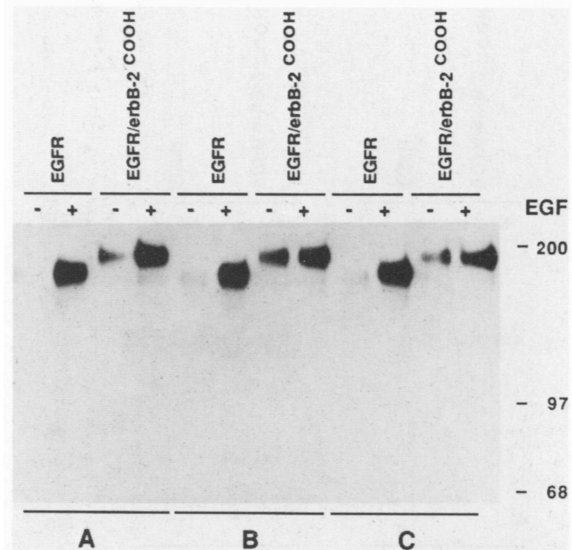

FIG. 5. In vitro autophosphorylation of EGFR and EGFR/*erbB-2*^{COOH}. Purified membranes from NIH 3T3 cells transfected with the indicated DNAs were obtained in the absence of phosphatase inhibitors, as described in Materials and Methods. Under these conditions, EGFR and EGFR/*erbB-2*^{COOH} were demonstrated, by immunoblot, to be completely depleted of PTyr (results not shown). The autophosphorylation assay was then performed in the presence of 60 μM cold ATP and 10 mM $MgCl_2$ under the indicated conditions. PTyr-containing proteins were analyzed by SDS-PAGE followed by immunoblot and detected with the anti-PTyr antibody. Molecular mass markers are indicated in kilodaltons. Comparable results were obtained in two independent experiments. The assay conditions utilized were as follows: 4°C for 10 min in the presence of 250 mM ammonium sulfate (A); 25°C for 1 min in the presence of 250 mM ammonium sulfate (B); and 4°C for 10 min without ammonium sulfate (C). Treatment with (+) or without (−) EGF was done as described in Materials and Methods.

EGFR/*erbB-2*^{COOH}, respectively. PLC-γ was not detectably phosphorylated on tyrosine residues in unstimulated NIH 3T3-EGFR (Fig. 6A). Following EGF treatment, PTyr-containing PLC-γ was readily detected. In accordance with the level of constitutive tyrosine phosphorylation of the EGFR/*erbB-2*^{COOH} molecule, constitutive levels of tyrosine-phosphorylated PLC-γ in NIH 3T3 cells overexpressing this chimera were observed (Fig. 6A). PLC-γ tyrosine phosphorylation could be further enhanced by the addition of EGF to these cells (Fig. 6A). Since no differences in the steady-state levels of PLC-γ could be detected in the two cell lines (Fig. 6B), we conclude that the EGFR/*erbB-2*^{COOH} chimera constitutively transduces an EGF-independent signal in vivo and that this activity correlates with its increased biological potency, compared with that of the EGFR.

DISCUSSION

erbB-2 and EGFR are closely related genes (6, 46) which have been implicated in the genesis of a variety of naturally occurring (11, 24, 26, 40, 42, 43, 53) and experimentally induced (2, 37) malignancies. In vitro studies have demonstrated that a number of heterogeneous genetic alterations, including overexpression (7, 8, 20), point mutations (2, 3, 39), and truncations (3, 8, 15), can activate the transforming potential of both molecules, likely through enhancement of receptor kinase-dependent intracellular signalling (4, 39, 45). There appears to be a fundamental difference in the consti-

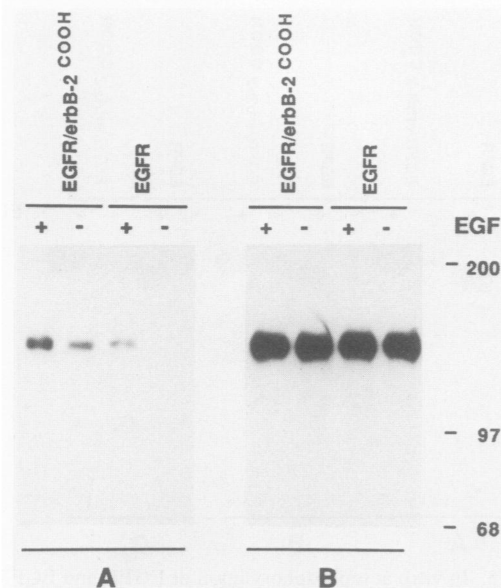

FIG. 6. In vivo tyrosine phosphorylation of PLC-γ by EGFR and EGFR/*erbB-2*^{COOH}. Two marker-selected mass cell populations expressing 1.5×10^6 and 1.3×10^6 EGFR and EGFR/*erbB-2*^{COOH} cells, respectively, were utilized. Cells were treated with EGF (100 ng/ml) at 37°C for 5 min, where indicated. (A) Total cell lysates (1.0 mg) were immunoprecipitated with the affinity-purified polyclonal antiPTyr antibody. Immunoprecipitates were then analyzed by immunoblot with a mixture of six monoclonal antibodies against PLC-γ (kindly provided by S. G. Rhee). (B) Total cell lysates (50 μg) were analyzed by immunoblot with the anti-PLC-γ antibodies described above. Molecular mass markers are indicated in kilodaltons. Comparable results were obtained in three independent experiments.

tutive level of tyrosine kinase activity attributed to steady-state expression of normal EGFR and *erbB-2* proteins. In this study, we endeavored to systematically analyze the effects of their different COOH domains on the biological and biochemical properties of *erbB-2* and EGFR molecules.

We showed that *erbB-2* and EGFR COOH domains differentially regulate their biological activities. By analysis of carboxy-terminal truncated mutants and chimeric molecules, we demonstrated that the *erbB-2* COOH domain significantly up regulates its biological activity. Thus, a COOH-truncated mutant demonstrated a reduction of transforming activity of as much as 40-fold. Moreover, substitution of the *erbB-2* COOH domain for its analogous domain in the EGFR resulted in a significant increase in EGFR-transforming activity in both the presence and absence of EGF. The mechanism by which its COOH domain exerts a positive regulatory role on *erbB-2* biological activity is not yet known. This domain may be important in the regulation of intrinsic receptor kinase activity or for interaction with critical substrates. The latter hypothesis seems less likely, since representative COOH-truncated *erbB-2* proteins demonstrated similar patterns of phosphorylation of intracellular proteins compared with those of the wild-type molecule. However, the fact that such mutants were less efficient suggests that COOH deletion causes a reduction in kinase activity in vivo.

Direct evidence that the *erbB-2* COOH domain up regulates kinase activity was derived from the replacement of the COOH domain of EGFR with the analogous domain of *erbB-2*. The EGFR/*erbB-2*^{COOH} chimeric protein demonstrated an increase in both in vivo and in vitro basal autophosphorylation activities, compared with the parental EGFR molecule. In contrast to the EGFR, the EGFR/*erbB-2*^{COOH} chimera constitutively phosphorylated PLC-γ on tyrosine residues in vivo, thus linking its enhanced biological activity to a well-known mitogenic signal transduction pathway (29, 32, 48). These findings provide a mechanistic basis for the increased transforming efficiency of this chimera, compared with that of the EGFR. Similar positive effector roles have been recently reported for COOH domains of both the insulin receptor (28, 31) and the platelet-derived growth factor receptor (10).

This study indicates that the EGFR COOH domain is not a strong modulator of biological activity. The *erbB-2*/EGFR^{COOH} chimeric protein exhibited 20-fold reduced transforming activity compared with the parental *erbB-2* molecule but was only slightly more efficient than the *erbB-2*-truncated mutant, *erbB-2*$^{\Delta1050}$. These findings are in agreement with previous reports showing that COOH truncation mutants of the EGFR displayed only a modest three- to fivefold reduction in their biological activities (15, 47).

The catalytic activity of the *erbB-2* product does not seem as tightly regulated as those of other growth factor receptors. In the absence of exogenously added ligand, *erbB-2* is capable of inducing transformation of cells from several different tissues (our unpublished observations) and possesses constitutive enzymatic activity (3, 39, 45; also this paper). A recent study has provided evidence for the existence of an *erbB-2* ligand (52). Thus, it is possible that the apparently atypical behavior of the *erbB-2* product might be due to autocrine production of such a ligand by a large array of normal cells. However, we showed that replacement of the EGFR COOH domain with *erbB-2* sequences alone was able to up regulate kinase activity and cause the EGFR/*erbB-2*^{COOH} molecule to display constitutive transforming activity. In this latter case, autocrine stimulation is highly unlikely, since NIH 3T3 cells do not express detectable tumor growth factor α or EGF (data not shown). Moreover, EGFR/*erbB-2*^{COOH} transformants maintained their transformed morphology in chemically defined medium lacking tumor growth factor α or EGF (data not shown). Thus, the *erbB-2* product may normally possess a high basal level of constitutive enzymatic activity in the absence of ligand at least in part because of the positive effector role of its COOH domain.

ACKNOWLEDGMENT

We are in debt to S. G. Rhee for providing the monoclonal antibodies against PLC-γ.

F. Fazioli is a recipient of an A.I.R.C. fellowship.

LITERATURE CITED

1. **Aaronson, S. A., and G. J. Todaro.** 1968. SV40 T antigen induction and transformation in human fibroblast cell strains. Virology **36**:254–261.
2. **Bargmann, C. I., M. C. Hung, and R. A. Weinberg.** 1986. Multiple independent activations of the *neu* oncogene by a point mutation altering the transmembrane domain of p185. Cell **45**:649–657.
3. **Bargmann, C. I., and R. A. Weinberg.** 1988. Oncogenic activation of the neu-encoded receptor protein by point mutation and deletion. EMBO J. **7**:2043–2052.
4. **Bargmann, C. I., and R. A. Weinberg.** 1988. Increased tyrosine kinase activity associated with the protein encoded by the activated neu oncogene. Proc. Natl. Acad. Sci. USA **85**:5394–5398.
5. **Chen, W. S., C. S. Lazar, M. Poenie, R. Y. Tsien, G. N. Gill, and**

M. G. Rosenfeld. 1987. Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. Nature (London) 328:820–823.

6. Coussens, L., T. L. Yang-Feng, Y. C. Liao, E. Chen, A. Gray, J. McGrath, P. H. Seeburg, T. A. Libermann, J. Schlessinger, U. Francke, and A. Ullrich. 1985. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science 230:1132–1139.
7. Di Fiore, P. P., J. H. Pierce, T. P. Fleming, R. Hazan, A. Ullrich, C. R. King, J. Schlessinger, and S. A. Aaronson. 1987. Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH 3T3 cells. Cell 51:1063–1070.
8. Di Fiore, P. P., J. H. Pierce, M. H. Kraus, O. Segatto, C. R. King, and S. A. Aaronson. 1987. *erb*B-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science 237:178–182.
9. Di Marco, E., J. H. Pierce, T. P. Fleming, M. H. Kraus, C. J. Molloy, S. A. Aaronson, and P. P. Di Fiore. 1989. Autocrine interaction between TGFa and the EGF-receptor: quantitative requirements for induction of the malignant phenotype. Oncogene 4:831–838.
10. Escobedo, J. A., P. J. Barr, and L. T. Williams. 1988. Role of tyrosine kinase and membrane-spanning domains in signal transduction by the platelet-derived growth factor receptor. Mol. Cell. Biol. 8:5126–5131.
11. Fukushige, S.-I., K.-I. Matsubara, M. Yoshida, M. Sasaki, T. Suzuki, K. Semba, K. Toyoshima, and T. Yamamoto. 1986. Localization of a novel v-*erb*B-related gene, c-*erb*B-2, on human chromosome 17 and its amplification in a gastric cancer cell line. Mol. Cell. Biol. 6:955–958.
12. Gill, G. N., P. J. Bertics, and J. B. Santon. 1987. Epidermal growth factor and its receptor. Mol. Cell. Endocrinol. 51: 169–186.
13. Graham, F. L., and A. J. Van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456–467.
14. Guroff, G. 1987. Oncogenes and growth factors. John Wiley & Sons, Inc., New York.
15. Haley, J. D., J. J. Hsuan, and M. D. Waterfield. 1989. Analysis of mammalian fibroblast transformation by normal and mutated human EGF receptors. Oncogene 4:273–283.
16. Hanks, S. K., A. M. Quinn, and T. Hunter. 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science 241:42–52.
17. Hari, J., and R. A. Roth. 1987. Defective internalization of insulin and its receptor in cells expressing mutated insulin receptors lacking kinase activity. J. Biol. Chem. 262:15341–15344.
18. Honegger, A. M., T. J. Dull, S. Felder, E. Van Obberghen, F. Bellot, D. Szapary, A. Schmidt, A. Ullrich, and J. Schlessinger. 1987. Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. Cell 51:199–209.
19. Honegger, A. M., D. Szapary, A. Schmidt, R. Lyall, E. Van Obberghen, T. J. Dull, A. Ullrich, and J. Schlessinger. 1987. A mutant epidermal growth factor receptor with defective protein tyrosine kinase is unable to stimulate proto-oncogene expression and DNA synthesis. Mol. Cell. Biol. 7:4568–4571.
20. Hudziak, R. M., J. Schlessinger, and A. Ullrich. 1987. Increased expression of the putative growth factor receptor p185HER2 causes transformation and tumorigenesis of NIH 3T3 cells. Proc. Natl. Acad. Sci. USA 84:7159–7163.
21. Kahn, P., and T. Graf. 1986. Oncogenes and growth control. Springer-Verlag, New York.
22. Kashles, O., D. Szapary, F. Bellot, A. Ullrich, J. Schlessinger, and A. Schmidt. 1988. Ligand-induced stimulation of epidermal growth factor receptor mutants with altered transmembrane regions. Proc. Natl. Acad. Sci. USA 85:9567–9571.
23. King, C. R., I. Borrello, F. Bellot, P. Comoglio, and J. Schlessinger. 1988. EGF binding to its receptor triggers a rapid tyrosine phosphorylation of the *erb*B-2 protein in the mammary tumor cell line SK-BR-3. EMBO J. 7:1647–1651.
24. King, C. R., M. H. Kraus, and S. A. Aaronson. 1985. Amplification of a novel v-*erb*B-related gene in a human mammary carcinoma. Science 229:974–976.
25. Kokai, Y., K. Dobashi, D. B. Weiner, J. N. Myers, P. C. Nowell, and M. I. Greene. 1988. Phosphorylation process induced by epidermal growth factor alters the oncogenic and cellular neu (NGL) gene products. Proc. Natl. Acad. Sci. USA 85:5389–5393.
26. Kraus, M. H., N. C. Popescu, S. C. Amsbaugh, and C. R. King. 1987. Over-expression of the EGF receptor-related proto-oncogene *erb*B-2 in human mammary tumor cell lines by different molecular mechanisms. EMBO J. 6:605–610.
27. Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA 82:488–492.
28. Maegawa, H., D. A. McClain, G. Freidenberg, J. M. Olefsky, M. Napier, T. Lipari, T. J. Dull, J. Lee, and A. Ullrich. 1988. Properties of a human insulin receptor with a COOH-terminal truncation. II. Truncated receptors have normal kinase activity but are defective in signaling metabolic effects. J. Biol. Chem. 263:8912–8917.
29. Margolis, B., S. G. Rhee, S. Felder, M. Mervic, R. Lyall, A. Levitzki, A. Ullrich, A. Zilberstein, and J. Schlessinger. 1989. EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signaling. Cell 57:1101–1107.
30. Martin, G. S. 1986. The *erb*B gene and the EGF receptor. Cancer Surv. 5:199–219.
31. McClain, D. A., H. Maegawa, J. Levy, T. Huecksteadt, T. J. Dull, J. Lee, A. Ullrich, and J. M. Olefsky. 1988. Properties of a human insulin receptor with a COOH-terminal truncation. I. Insulin binding, autophosphorylation, and endocytosis. J. Biol. Chem. 263:8904–8911.
32. Meisenhelder, J., P.-G. Suh, S. G. Rhee, and T. Hunter. 1989. Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases *in vivo* and *in vitro*. Cell 57:1109–1122.
33. Moolenaar, W. H., A. J. Bierman, B. C. Tilly, I. Verlaan, L. H. Defize, A. M. Honegger, A. Ullrich, and J. Schlessinger. 1988. A point mutation at the ATP-binding site of the EGF-receptor abolishes signal transduction. EMBO J. 7:707–710.
34. Mulligan, R. C., and P. Berg. 1981. Selection for animal cells that express the Escherichia coli gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. USA 78:2072–2076.
35. Pang, D. T., B. R. Sharma, and J. A. Shafer. 1985. Purification of the catalytically active phosphorylated form of insulin receptor kinase by affinity chromatography with O-phosphotyrosyl-binding antibodies. Arch. Biochem. Biophys. 242:176–186.
36. Pruss, R. M., and H. R. Herschman. 1977. Variants of 3T3 cells lacking mitogenic response to epidermal growth factor. Proc. Natl. Acad. Sci. USA 74:3918–3921.
37. Schechter, A. L., D. F. Stern, L. Vaidyanathan, S. J. Decker, J. A. Drebin, M. I. Greene, and R. A. Weinberg. 1984. The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. Nature (London) 312:513–516.
38. Schlessinger, J. 1986. Allosteric regulation of the epidermal growth factor receptor kinase. J. Cell Biol. 103:2067–2072.
39. Segatto, O., C. R. King, J. H. Pierce, P. P. Di Fiore, and S. A. Aaronson. 1988. Different structural alterations upregulate in vitro tyrosine kinase activity and transforming potency of the *erbB*-2 gene. Mol. Cell. Biol. 8:5570–5574.
40. Semba, K., N. Kamata, K. Toyoshima, and T. Yamamoto. 1985. A v-*erb*B-related proto-oncogene, c-*erb*B-2, is distinct from the c-*erb*B-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma. Proc. Natl. Acad. Sci. USA 82:6497–6501.
41. Sherr, C. J., and C. W. Rettenmier. 1986. The fms gene and the CSF-1 receptor. Cancer Surv. 5:221–232.
42. Slamon, D. J., G. M. Clark, S. G. Wong, W. J. Levin, A. Ullrich, and W. L. McGuire. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 235:177–182.
43. Slamon, D. J., W. Godolphin, L. A. Jones, J. A. Holt, S. G. Wong, D. E. Keith, W. J. Levin, S. G. Stuart, J. Udove, A.

Ullrich, and M. F. Press. 1988. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science **244**:707–712.

44. **Stern, D. F., and M. P. Kamps.** 1988. EGF-stimulated tyrosine phosphorylation of p185neu: a potential model for receptor interactions. EMBO J. **7**:995–1001.
45. **Stern, D. F., M. P. Kamps, and H. Cao.** 1988. Oncogenic activation of p185*neu* stimulates tyrosine phosphorylation in vivo. Mol. Cell. Biol. **8**:3969–3973.
46. **Ullrich, A., L. Coussens, J. S. Hayflick, T. J. Dull, A. Gray, A. W. Tam, J. Lee, Y. Yarden, T. A. Libermann, J. Schlessinger, J. Downward, E. L. V. Mayes, N. Whittle, M. D. Waterfield, and P. H. Seeburg.** 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature (London) **309**:418–425.
47. **Velu, T. J., W. C. Vass, D. R. Lowy, and L. Beguinot.** 1989. Functional heterogeneity of proto-oncogene tyrosine kinases: the C terminus of the human epidermal growth factor receptor facilitates cell proliferation. Mol. Cell. Biol. **9**:1772–1778.
48. **Wahl, M. I., N. E. Olashaw, S. Nishibe, S. G. Rhee, W. J. Pledger, and G. Carpenter.** 1989. Platelet-derived growth factor induces rapid and sustained tyrosine phosphorylation of phospholipase C-λ in quiescent BALB/c 3T3 cells. Mol. Cell. Biol. **9**:2934–2943.
49. **Wigler, M., S. Silverstein, L.-S. Lee, A. Pellicer, Y.-C. Cheng, and R. Axel.** 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell **11**:223–232.
50. **Yarden, Y., J. A. Escobedo, W. J. Kuang, T. L. Yang-Feng, T. O. Daniel, P. M. Tremble, E. Y. Chen, M. E. Ando, R. N. Harkins, U. Francke, V. A. Fried, A. Ullrich, and L. T. Williams.** 1986. Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. Nature (London) **323**:226–232.
51. **Yarden, Y., W. J. Kuang, T. Yang-Feng, L. Coussens, S. Munemitsu, T. J. Dull, E. Chen, J. Schlessinger, U. Francke, and A. Ullrich.** 1987. Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J. **6**:3341–3351.
52. **Yarden, Y., and R. A. Weinberg.** 1989. Experimental approaches to hypothetical hormones: detection of a candidate ligand of the *neu* protooncogene. Proc. Natl. Acad. Sci. USA **86**:3179–3183.
53. **Yokota, J., T. Yamamoto, K. Toyoshima, M. Terada, T. Sugimura, H. Battifora, and M. J. Cline.** 1986. Amplification of c-*erb*B-2 oncogene in human adenocarcinomas in vivo. Lancet **i**:765–767.