Domain-specific Interactions between the p185<sub>neu</sub> and Epidermal Growth Factor Receptor Kinases Determine Differential Signaling Outcomes

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We expressed the epidermal growth factor receptor (EGFR) along with mutant p185<sub>neu</sub> proteins containing the rat transmembrane point mutation. The work concerned the study of the contributions made by various p185<sup>neu</sup> subdomains to signaling induced by a heterodimeric ErbB complex. Co-expression of full-length EGFR and oncogenic p185<sup>neu</sup> receptors resulted in an increased EGFR-dependent phosphorylation of p185<sup>neu</sup>, increased cell proliferation to limiting concentrations of EGF, and increases in both EGF-induced MAPK and phosphatidylinositol 3-kinase (PI 3-kinase) activation. Intracellular domain-deleted p185<sup>neu</sup> receptors (T691stop neu) were able to associate with full-length EGFR, but induced antagonistic effects on EGFR-dependent EGFR receptor down-regulation, cell proliferation, and activation of MAPK and PI 3-kinase pathways. Ectodomain-deleted p185<sup>neu</sup> proteins (TΔ5) were unable to physically associate with EGFR, and extracellular domain-deleted p185<sup>neu</sup> forms failed to augment activation of MAPK and PI 3-kinase in response to EGF. Association of EGFR with a carboxyl-terminally truncated p185<sup>neu</sup> mutant (TAPstop neu) did not increase transforming efficiency and phosphotyrosine content of the TAPstop species, and proliferation of EGFR/TAPstop-co-expressing cells in response to EGF was similar to cells containing EGFR only. Thus, neither cooperative nor inhibitory effects were observed in cell lines co-expressing either TΔ5 or TAPstop mutant proteins. Unlike the formation of potent homodimer assemblies composed of oncogenic p185<sup>neu</sup>, the induction of signaling from p185<sup>neu</sup>-EGFR heteroreceptor assemblies requires the ectodomain for ligand-dependent physical association and intracellular domain contacts for efficient intermolecular kinase activation.

The ErbB family includes four members of homologous receptor tyrosine kinases, the epidermal growth factor receptor (EGFR) or ErbB-1 (1), ErbB-2-p185<sup>neu</sup> (2, 3), ErbB-3 (4), and ErbB-4 (5). ErbB family proteins are widely expressed in epithelial, mesenchymal, and neuronal tissues, and play important roles in normal growth and development (6–9). Aberrant expression of these ErbB proteins is frequently observed in human malignancies (10).

The transmembrane mutation in rat p185<sup>neu</sup> (also termed Neu) (12) serves as a paradigm for receptor dimerization that leads to constitutive kinase activation contributing to oncogenic transformation (11–13). Additional support for this mechanism has come from the identification of a naturally occurring activated EGFR oncprotein (ΔEGFR or EGFRvIII) in human tumors, which forms constitutive dimers and confers increased tumorigenicity (14, 15). Gene amplification and overexpression of ErbB-2 have been observed in a high frequency of human adenocarcinomas, including those of the breast and ovary, and these features correlate with poor clinical prognosis (16, 17). Experimental support for this model is provided by in vitro transformation assays using cell lines overexpressing either protooncogenic rat p185<sup>neu</sup> or human ErbB-2 at levels of 10<sup>6</sup> receptors/cell (18, 19). Biochemical and biophysical analysis of baculovirus-expressed p185<sup>neu</sup> proteins further support the notion of receptor oligomerization as a mechanism of kinase activation of normal holoreceptors (20, 21).

Heterodimeric interactions govern many signaling properties within the ErbB receptor family. Co-expression of EGFR and p185<sup>neu</sup> at modestly elevated levels (10<sup>5</sup>/cell) (but not either receptor independently) results in synergistic transactivation (22), due to increase of the ligand binding affinity and catalytic kinase activity (23, 24). Heterodimerization of EGFR and ErbB-2 has also been observed in human breast tumor lines (25). Moreover, ligand treatment promotes the assembly of an activated p185<sup>neu</sup>-EGFR kinase complex in many cells (24), resulting in novel distinct cellular signaling events (26). Therefore, the receptor tyrosine kinase ensemble can be activated not only by homodimer formation, but also by heterodimeric associations. In this regard, endodomain interactions between p185<sup>neu</sup> and EGFR appear to influence...
functional signaling outcomes (27).

In response to EGF or Neu differentiating factor/heregulin (a ligand for ErbB-3 and ErbB-4) family ligands (28, 29), EGFR and ErbB-2 both form heterodimers with ErbB-3 and ErbB-4 (30–34). Heterodimers between p185 Neu/ErbB-2 and ErbB-3 are associated with activated signaling and the transformed phenotype in primary human cancer cells (35). Existence of an ErbB-3-ErbB-4 heterodimer has not been convincingly demonstrated to date. More recent data support the notion that p185 Neu/ErbB-2 is the preferred heterodimerization partner of all ErbB receptors and a mediator for divergent cellular signaling in many distinct cell types (34, 36).

The structural basis for ErbB receptor heterodimerization has not been completely defined and crystallographic information on dimerized ErbB receptor kinases is currently unavailable. Previous work has revealed that ectodomain interactions are sufficient to stabilize dimer formation between p185 Neu and EGFR in fibroblasts and transformed cells (5, 37, 38), which is supported by observations showing that a partial deletion of the EGF receptor ectodomain still allows dimer formation and receptor activation (14, 15). Although the transmembrane domain alone can stabilize the formation of p185 Neu homodimers, the ectodomain has not been directly compared regarding the formation of signaling heterodimers.

In this study, we have constructed various p185 Neu deletion mutants in order to specifically compare signaling events resulting from associations between EGFR receptors and either p185 Neu ectodomain- or endodomain-derived mutant receptors. We have co-expressed EGFR with low levels of p185 Neu proteins, or their mutant derivatives, to monitor p185 Neu-mediated enhancement of cell growth and transformation in vitro and in vivo, and to analyze the influence of EGF-induced heterodimeric receptor interactions on downstream signaling effectors. Signaling resulting from heterodimeric associations between full-length EGFR and mutant p185 Neu proteins has revealed the functional importance of p185 Neu subdomains in the induction of Ras/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI 3-kinase) pathways contributing to cell growth and transformation.

**Experimental Procedures**

**Antibodies—**As described previously (20, 39, 40), monoclonal antibody 7.16.4, polyclonal antiserum a-Bacneu, and NCT are reactive with the ectodomain, intracellular domain, and carboxyl terminus of p185 Neu, respectively. mAb 225 reactive with the ectodomain of EGFR was obtained from Dr. John Mendelsohn (M. D. Anderson Cancer Center, Dallas, TX). A polyclonal rabbit antiserum specifically against the COOH terminus of EGFR (termed CT) was provided by Dr. Stuart Decker (40). The anti-phosphotyrosine monoclonal antibody, PY20, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA.).

**DNA Constructs—**All the deletion mutants were derived from the rat oncogenic p185 Neu cDNA containing a single point mutation (V664G) in the transmembrane domain. The TAPstop mutant, containing a 122-aa deletion (42, 43). The ectodomain-deleted mutant TAPstop was co-transfected with pSR constructs into murine fibroblasts. These wild-type or mutant p185 Neu forms were all cloned into the pSV2neo/DHFR vector as described (44).

**Transfection and Maintenance of Cell Lines—**Ten micrograms of the p185 Neu constructs were transfected into NR6 cells, a mouse fibroblast cell line devoid of endogenous EGFR receptors (43), or NE91 cells expressing human EGFR (37) by calcium phosphate precipitation. After 2–3 weeks of selection with Genetin (0.9 mg/ml), the established stable clones were screened and characterized. Gene amplification by methotrexate was used to increase the p185 Neu receptor level. Expression of p185 Neu and its derivatives in resultant subclones was examined by flow cytometric analysis following anti-p185 Neu mAb 7.16.4 staining. Surface expression of p185 Neu proteins was then estimated by comparing the mean channel fluorescence intensity with that of B104-1-1 cells, a low level of p185 Neu in NR6-1 cells was provoked by stable transfection with 125I-labeled anti-neu mAb binding assay (22). EGFR numbers in NE91 cells and mutant p185 Neu co-transfected cells were determined by Scatchard assays as described (37). These transfected clones were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal bovine serum (FBS, HyClone) at 37 °C in a 5% CO2 atmosphere.

**Cross-linking, Immunoprecipitation, and Immunoblotting Procedures—**Cells in 70- to 80-mm dishes were washed and stained in cysteine-free DMEM for 1 h, and grown in low cysteine-containing 5% FBS-DMEM containing 55 μCi/ml [35S]Sulfate (Amersham Pharmacia Biotech) for 16 h for metabolic labeling. Alternatively, the unlabeled cells were cultured overnight in 10-cm Petri dishes. After treatment with or without EGF, cells were washed twice with cold phosphate-buffered saline (PBS) and treated with PBS containing 2 mM membrane-impermeable cross-linker bisulfosuccinimidyl) suberate (BS3, Pierce), for 30 min. After quenching the cross-linking reaction with a buffer containing 10 mM Tris-HCl (pH 7.6), 0.9% NaCl, and 0.1 mM glycine, cells were washed twice with cold PBS and solubilized with PU/RIPA buffer as described (24). The immunocomplexes were washed and solubilized, then separated by gradient SDS-PAGE gels (4–7.5%). Proteins in cell lysates were analyzed by autoradiography. Proteins from unlabelled cells were transferred onto nitrocellulose and then immunoblotted with anti-phosphotyrosine mAb PY20, anti-EGFR CT, or anti-p185 antisem as indicated in the figure. The protein signals were identified by the binding of 125I-labeled protein A (NEN Life Science Products), or by enhanced chemiluminescence (ECL) using ECL kit from Amersham Pharmacia Biotech.

**Receptor Down-regulation Studies—**Cells (1 x 10⁶) were plated in a six-well dish with DMEM containing 5% FBS overnight. Cells were then treated with EGF (50 ng/ml) for 0–4 h and were harvested and washed with cold PBS containing 0.5% bovine serum albumin and 0.1% sodium azide. Cell preparations were then incubated with a saturating amount (0.5 μg/reaction) of anti-neu mAb 7.16.4 or anti-EGFR mAb 225, or an irrelevant mAb (such as 9BG5 against the hemagglutinin of influenza virus receptor), for 4°C for 30 min, restained with fluorescein isothiocyanate-conjugated anti-mouse IgG (Sigmagamma) for another 30 min after extensive washing. Cells were then fixed with 2% paraformaldehyde and analyzed by flow cytometry (FACScan, Becton Dickinson), as described previously (37). Briefly, after subtracting the nonspecific background staining with 9BG5, the mean channel values from each time point were used to determine the percentage of surface expression of EGFR or p185 Neu proteins at the various time points after EGF treatment.

**In Vitro and In Vivo Transformation Assays—**Anchorage-independent growth ability was determined by assessing the colony forming efficiency of cells suspended in soft agar (15, 37). Cells (1000/dish) were suspended in 7% FBS-DMEM containing 0.1% agarose, and plated on 0.25% basal agar in each dish. Cells were fed three times with 5% FBS-DMEM containing 55 Ci/ml [35S]cysteine (Amersham Pharmacia Biotech) for 16 h for metabolic labeling. Alternatively, the unlabeled cells were cultured overnight in 10-cm Petri dishes. After treatment with or without EGF, cells were washed twice with cold phosphate-buffered saline (PBS) and treated with PBS containing 2 mM membrane-impermeable cross-linker bisulfosuccinimidyl) suberate (BS3, Pierce), for 30 min. After quenching the cross-linking reaction with a buffer containing 10 mM Tris-HCl (pH 7.6), 0.9% NaCl, and 0.1 mM glycine, cells were washed twice with cold PBS and solubilized with PU/RIPA buffer as described (24). The immunocomplexes were washed and solubilized, then separated by gradient SDS-PAGE gels (4–7.5%). Proteins in cell lysates were analyzed by autoradiography. Proteins from unlabelled cells were transferred onto nitrocellulose and then immunoblotted with anti-phosphotyrosine mAb PY20, anti-EGFR CT, or anti-p185 antisem as indicated in the figure. The protein signals were identified by the binding of 125I-labeled protein A (NEN Life Science Products), or by enhanced chemiluminescence (ECL) using ECL kit from Amersham Pharmacia Biotech.

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**EFGD-dependent Cell Proliferation Assay—**The 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay for measuring cell growth has been described previously (38). Briefly, cells (3000/well) of each cell line were seeded in 96-well plates overnight in DMEM containing 5% FBS. Cells were then washed in serum-free ITS-DMEM for 48 h, then cultured in 100 μl of the same medium, plus various concentrations of EGF for another 48 h. 25 μl of MTT solution (5 μg/ml in PBS) were added to each well, and after 2 h of incubation at 37 °C, 100 μl of the extraction buffer (20% w/v SDS, 50% N,N-dimethyl formamide, pH 4.7) was added. After an overnight incubation at 37 °C, the optical density at 600 nm was measured using an enzyme-linked immunosorbent assay reader. Each value represents a mean of four samples.
MAP Kinase and PI 3-Kinase Immune Complex Kinase Assays—

COS7 cells were transiently transfected with pcDNA3-HA-ERK2 (a gift from Silvio Gutkind, National Institutes of Health, Bethesda, MD) and pShuEGFR/hyg, along with either empty vector or plasmids expressing wild-type or mutant p185 neu using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions and assayed 48 h after transfection. Cells deprived of serum for 16–20 h were treated with or without EGF (50 ng/ml) for 5 min. For MAP kinase assay, cells were lysed with RIPA buffer (25 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 150 mM NaCl, 1 mM MgcI, 2 mM EDTA, 0.5 mM dithiothreitol, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonfluoride, 10 μg/ml leupeptin). Protein concentrations were determined by the BCA kit (Pierce). Equal amounts of protein (100 μg) from cell extracts were immunoprecipitated with anti-
ti-HA (BabCo). After washing extensively, the immunocomplexes were then incubated with 50 μl of reaction buffer (30 mM HEPES (pH 7.4), 10 mM MgCl2, 1 mM dithiothreitol, 5 μM ATP) containing 1 μCi of [γ-32P]ATP (NEN Life Science Products) and 2 μg of myelin basic protein (Upstate Biotechnology Inc.). After incubation for 20 min at 30 °C, kinase reactions were terminated by the addition of 2× Laemmli sample buffer. The samples were then resolved by SDS-PAGE, and the phosphorylated myelin basic protein was visualized by autoradiography. PI 3-kinase immune assays were carried out as described (45). The autoradiograms were scanned by a densitometer (Molecular Dynamics, Sunnyvale, CA) and relative expression levels of various p185 neu mutant proteins in selected clones were estimated by a comparison with B104-1-1 cells, while the expression of EGFR in these cells was estimated by Scatchard analysis. In order to observe an enhancement of EGFR-mediated cellular signaling and transformation, clone Er/p185 neu expressing a moderately low level of both receptors (~10^3/cell) was chosen. In other subclones, the expression of EGFR and/or mutant p185 neu proteins was approximately ~10^6 receptors/cell.

The Ectodomain of p185 neu Is Required for Heterodimerization with EGFR—

Stable cell lines expressing EGFR and/or mutant p185 neu proteins were used to assess dimer formation using the chemical cross-linker BS3. As shown in Fig. 2, B104-1-1 cells expressing oncogenic p185 neu contained p185 neu homodimers (~370 kDa) independent of ligand stimulation (Fig. 2a, lane 1), due to the activating transmembrane mutation (12). A cell line expressing the ectodomain-derived T691stop neu alone was used as a control to demonstrate the sizes of the monomer and dimer of this truncated p185 neu protein, which migrated at approximately 115 kDa (Fig. 2a, lanes 2 and 3), and at ~230 kDa in the presence of a chemical cross-linker (Fig. 2a, lane 3).

In the presence of EGF, the 170-kDa monomeric form and the 340 kDa homodimer of EGFR were both detected in NE91 cells expressing EGFR alone, and in ErT91stop cells (Fig. 2a, lanes 4 and 5, respectively). An additional intermediate band of ~285 kDa representing the heterodimer of EGFR and T91stop was clearly detectable upon anti-EGFR immunoprecipitation (Fig. 2a, lane 5). The 285-kDa intermediate complex was similar to the heterodimer composed of EGFR and truncated N91stop derived from proto-oncogenic p185 neu as described previously (44), except that the heterodimeric EGFR-N91stop complex was even more predominant than the EGFR-N91stop mimer. In these studies, Notably, T691stop is still able to complex with EGFR (lane 5) even under conditions favorable for T691stop homodimerization (lane 3). Densitometric analysis suggested that at least 50% of the EGFR associated with T691stop neu in a heterodimeric complex in ErT91stop cells (Fig. 2a, lane 5), further suggesting the strong preference for EGFR:p185 neu heterodimerization.

We have previously studied complex formation between the p185 neu and EGFR holoreceptors (22, 24, 37) and heterodimeric.
The number of EGFR on NE91 and other transfected cells was determined by Scatchard assays. Cell surface expression of neu proteins were estimated by comparing the mean channel fluorescent intensity with that from B104–1-1 cells using flow cytometry analysis. p185<sup>neo</sup> on B104–1-1 cells was originally determined by an ¹²⁵I-labeled anti-neu mAb binding assay (22). For the tumor growth assay, individual clones (1 x 10<sup>6</sup> cells/site) were injected intradermally into athymic mice. NT, no tumor after 10 weeks; ND, not determined.

| Cells          | Colony in soft agar | Incidence | Latency | mm<sup>3</sup> (at week 6) | Receptor expression |
|----------------|---------------------|-----------|---------|----------------------------|---------------------|
|               | % efficiency        |           |         |                            | Neu protein | EGFR |
| B104–1-1      | 33.7 ± 0.6          | 6/6       | 1       | 1.5 x 10<sup>6</sup>       | 0          | 0    |
| NE91          | <0.1                | 0/4       | NT      | 0                          | 2.8 x 10<sup>4</sup> |
| T<sub>neu</sub>| 5.4 ± 0.4           | 4/4       | 4–5     | 3.7 x 10<sup>4</sup>       | 0          | 0    |
| Er<sub>T</sub>neu | 10.2 ± 0.6         | 6/6       | 2–5     | 3.9 x 10<sup>4</sup>       | 3.8 x 10<sup>4</sup> |
| T<sub>691stop</sub>| <0.1                | 0/4       | NT      | 4.5 x 10<sup>3</sup>       | 0          | 0    |
| Er/ T<sub>691stop</sub> | <0.1              | 0/4       | NT      | 4.3 x 10<sup>3</sup>       | 2.5 x 10<sup>5</sup> |
| TAP<sub>stop</sub>| 6.5 ± 0.5           | 6/6       | 4–5     | 1.2 x 10<sup>5</sup>       | 0          | 0    |
| Er/TAP<sub>stop</sub> | 5.5 ± 0.6          | 6/6       | 4–5     | 1.3 x 10<sup>5</sup>       | 4.6 x 10<sup>5</sup> |
| T<sub>Δ5</sub> | 5.7 ± 0.3           | 6/6       | 3       | 545.7                      | ND         | 0    |
| Er/T<sub>Δ5</sub> | 6.6 ± 0.4          | 4/4       | 3       | 593.4                      | ND         | 1.6 x 10<sup>5</sup> |

**Fig. 2. Homodimerization and heterodimerization of EGFR and p185<sup>neo</sup> proteins.** A, cells were labeled with [³⁵S]cysteine overnight. Cell lines expressing EGFR (lanes 4 and 5) were then stimulated with EGF (200 ng/ml) at 37 °C for 10 min. All cells (except lane 2) were treated with the chemical cross-linker BS<sub>3</sub> (2 mM). Cell lysates were then immunoprecipitated with anti-neu mAb 7.16.4 or anti-EGFR antiserum CT as indicated. Proteins were separated by 4–8% gradient SDS-PAGE and analyzed by autoradiography. The estimated molecular weight of monomers and dimers is indicated. B and C, cell lines expressing EGFR (NE91, Er/T<sub>Δ5</sub>, and Er/TAP<sub>stop</sub>) were stimulated with EGF. After BS<sub>3</sub> treatment, all the cells were lysed and subjected to immunoprecipitation with either anti-neu (7.16.4 or NCT) or anti-EGFR (CT) antibodies, then immunoblotted with either the anti-neu (NCT or α-Bacneu) or anti-EGFR probe (CT) as indicated.

Heterodimerization between EGFR and the ectodomain-deleted T<sub>Δ5</sub> p185<sup>neo</sup> mutant was also analyzed. T<sub>Δ5</sub> can be recognized by either the α-Bacneu or anti-NCT polyclonal antibodies reactive with the intracellular domain or carboxyl terminus of the p185<sup>neo</sup> protein, respectively. Immunoblotting showed that the size of the TΔ5 neu mutant was approximately 95–97 kDa, and the detectable dimeric form was about −200 kDa (Fig. 2B, lane 1). Er/T<sub>Δ5</sub> cells express a high level of EGFR and TΔ5, as homodimers of either form were clearly detected in the presence of cross-linker (Fig. 2B, lanes 2 and 3), when compared with control cell lines NE91 and TΔ5 (Fig. 2B, lanes 1 and 4). However, unlike Er/T<sub>691stop</sub> and Er/TAP<sub>stop</sub> cells, the heterodimer between EGFR and TΔ5 in Er/TΔ5 cells was undetectable following EGF and BS<sub>3</sub> treatment since the predicted intermediate size (−270 kDa) complex representing EGFR and TΔ5 heterodimer was not observed (Fig. 2B, lanes 2 and 3). In an attempt to identify the association of EGFR with this ectodomain-deleted TΔ5 protein, several alternative assays were performed, such as using the membrane-permeable chemical cross-linker DSP (Pierce), or a mild detergent digitonin lysis buffer. These methods were sensitive enough to detect the complex formation between full-length p185<sup>neo</sup> and TΔ5 (27).
However, the association of EGFR and $\Delta 5$ was still undetectable (data not shown). Taken together, these results strongly suggest that the ectodomain of the p185$^{neu}$ receptor is necessary and sufficient for heterodimerization with holoreceptor EGFR.

**Tyrosine Kinase Activity in Living Cells**—It has been well documented that EGF, in an EGFR-dependent manner, stimulates phosphorylation of the p185$^{neu}$ and c-ErbB-2 gene products with a concomitant increase in their tyrosine kinase activities (46–49). Heterodimerization of p185 and EGFR facilitates cross-phosphorylation (24, 25), since a full-length, kinase-deficient p185$^{neu}$ mutant (K757M) is trans-phosphorylated when physically associated with EGFR (37). We next examined the tyrosine phosphorylation level of p185$^{neu}$ derivatives in living cells in response to EGF treatment. After the addition of EGF, oncogenic p185$^{neu}$ and its derivatives were immunoprecipitated with anti-neu antibodies, and receptor phosphotyrosine content in vivo was detected by immunoblotting with an anti-phosphotyrosine antibody (PY20) (Fig. 3). Full-length p185$^{neu}$ from control B104-1-1 fibroblasts displayed constitutive kinase activity (Fig. 3A, lane 1). Upon EGF stimulation, there was indeed an additional increase in tyrosine kinase activity of p185$^{neu}$ in Er/neu cells expressing lower amounts of the p185$^{neu}$ protein (Fig. 3A, lanes 4 and 5), but not in cells expressing p185$^{neu}$ alone (lanes 2 and 3). A weak tyrosine phosphorylation signal was detected in TAPstop cells (Fig. 3A, lane 6). EGF stimulation did not appreciably increase the tyrosine phosphorylation of TAPstop in EGFR-co-expressing cells (Fig. 3A, lanes 7 and 8), although the association of EGFR and TAPstop was evident (Fig. 3C). Truncation of the p185$^{neu}$ carboxyl terminus, and deletion of at least three known critical tyrosine residues, was associated with the failure to trans-phosphorylate the p185$^{neu}$ mutant protein. Elimination of the ectodomain did not impair the intrinsic kinase activity of p185$^{neu}$-derived $\Delta 5$, since the $\Delta 5$ mutant receptor was still a competent tyrosine kinase (Fig. 3C, lane 1). However, unlike the full-length p185$^{neu}$, no further increase in tyrosine phosphorylation of $\Delta 5$ was detected in Er/$\Delta 5$ cells with EGF stimulation (Fig. 3C, lane 2 and 3). In Er/$\Delta 5$ cells, the EGFR was also immunoprecipitated by the anti-Bacneu antisera and still autophosphorylated after EGF treatment (Fig. 3C, lane 3). These results correlated with failure to detect physical interactions between EGFR and $\Delta 5$ proteins (Fig. 2). Re-probing with anti-neu antibodies (Fig. 3, A and C) confirmed equivalent protein loading in paired samples with or without EGF treatment (Fig. 3, B and D). These experiments indicated that the full-length p185$^{neu}$ receptor, but not mutant p185$^{neu}$ proteins with NH$_2$-terminal or distal COOH-terminal truncations, was able to interact with activated EGFR functionally, resulting in trans-phosphorylation.

We next analyzed tyrosine kinase activation in EGFR-positive NE91 cells with or without T691stop neu co-expression. Treatment with EGF and a chemical cross-linking reagent resulted in heavy tyrosine phosphorylation of EGFR monomers and homodimers in NE91 cells (Fig. 3E, lane 1). No detectable tyrosine phosphorylation of cytoplasmic domain-deleted T691stop neu was seen in cells with or without EGF co-expression (Fig. 3E, lanes 2 and 3, respectively). In addition, the tyrosine phosphorylation signal of an intermediate band (285 kDa) representing EGFR-T691stop heterodimeric complex was also undetectable (Fig. 3E, lane 2), although a significant portion of EGFR forms a heterodimer with T691stop under these conditions (Fig. 2A, lane 5). Tyrosine kinase activation of full-length EGFR was thus completely inhibited when EGFR was physically associated with the T691stop neu mutant protein, which correlates with reduction of the transformed phenotype of primary EGFR-positive glioma cells expressing T691stop neu (42). Moreover, these results are consistent with the observation from cells co-expressing EGFR with N691stop neu derived from normal p185$^{neu}$ (37).

Re-probing the membrane with an anti-EGFR antibody (CT) showed total EGFR levels in NE91 cells (Fig. 3F, lane 1), and confirmed the presence of the EGFR-T691stop heterodimer (285 kDa), since this complex was recognized by anti-neu in...
immunoprecipitation and anti-EGFR in immunoblotting (Fig. 3F, lane 2). Lysates obtained from T691stop neu-expressing cells did not react with the anti-EGFR CT probe (Fig. 3F, lane 3). Although the cytoplasmic domain deletion in T691stop did not impair heterodimerization with EGFR, the undetectable phosphotyrosine content of the intermediate heterodimer suggested that EGFR kinase activity was reduced when associated with T691stop neu. These experiments further support our model that the heteroreceptor assembly mediated primarily by ectodomain interactions facilitates kinase trans-activation and trans-phosphorylation caused by interactions between cytoplasmic domains (15, 27, 37).

EGF-induced Receptor Down-regulation from the Cell Surface—Numerous studies indicate that ligand-mediated receptor endocytosis and degradation is a kinase-dependent process for many types of growth factor receptors (50). We found that the efficiency of receptor down-regulation and degradation in cells co-expressing EGFR and p185 neu correlated well with heterodimeric kinase activities (37). We used this method as an alternative assay to examine the kinase activity of various heterodimers.

Cells were incubated with EGF (50 ng/ml) for various times prior to cell surface staining with anti-neu mAb 7.16.4 or anti-EGFR mAb 225 followed by the staining with fluorescein isothiocyanate-conjugated anti-mouse-IgG. Cell surface expression of each receptor was analyzed using flow cytometric analysis. EGF treatment of NE91 cells (expressing EGFR only) resulted in a reduction of cell surface EGFR, and over 60% of EGFR receptors disappeared from the cell surface after 4 h of treatment (Fig. 4A). Normal EGFR down-regulation was not affected by the co-expression of T35, as the efficiency of EGFR down-regulation in Er/T35 cells was very similar to that seen in NE91 cells (Fig. 4A). A similar EGFR down-regulation curve was observed in Er/neu and Er/TAPstop cells (Fig. 4, C and D, respectively), indicating that the EGFR behaves as an active receptor kinase in these cells. Moreover, about ~20% of p185 neu or 25% TAPstop was co-down-regulated with EGFR upon EGF stimulation (Fig. 4, C and D). As illustrated above, the low expression of p185 neu and EGFR in Er/neu cells was insufficient to demonstrate the physical association of the two receptors biochemically. The current assay was more sensitive in determining EGF-mediated receptor interactions. Control cells expressing TAPstop alone did not respond to EGF treatment, and the surface expression of TAPstop remained unchanged within the period of EGF treatment (Fig. 4D).

Analysis using an EGF-mediated pulse-chase assay showed that the down-regulated EGFR and co-down-regulated TAPstop proteins efficiently went into the degradation pathway (data not shown), similar to the cells overexpressing EGFR and p185 neu (37). Our data suggested that EGFR and either p185 neu or TAPstop associated into an active kinase complex and that these receptor assemblies exhibited comparable kinetics of receptor endocytosis.

However, co-expression of T691stop with EGFR resulted in diminished EGF-induced down-regulation of EGFR. The maximal reduction of surface EGFR receptor was ~35% after 4 h. In addition, no detectable co-down-regulation of the cytoplasmic domain deleted T691stop was observed in Er/T691stop cells (Fig. 4B), correlating with the observation of the inactive heterodimer of EGFR/T691stop (Fig. 3, E and F). This finding supports the idea that receptor down-regulation is coupled to receptor tyrosine kinase activity. The formation of the inactive heterodimer between EGFR and T691stop neu proteins influenced the overall kinetics of EGFR down-regulation. Impairment of ligand-induced down-regulation of holo-EGFR by T691stop neu has also been observed in primary human cancer cells.

Transforming Potency of Cells Expressing Mutant p185 neu Proteins with or without EGFR—We and others have showed that the transforming potency of p185 neu requires not only its intrinsic tyrosine kinase activity (13), but also the crucial role.

2 D. M. O'Rourke and M. I. Greene, unpublished observations.
of tyrosine phosphorylation of its carboxyl terminus, as the oncogenicity of p185<sup>new</sup> was greatly reduced by alteration of several tyrosine residues (41) or large structural deletions, such as seen with TAP<sub>stop</sub> (42). Transforming ability of ectodomain-deleted TΔ5 in this system was less potent than full-length p185<sup>new</sup>, possibly due to the reduced efficiency of forming active receptor complexes when compared with full-length oncogenic p185<sup>new</sup> (27) (see Fig. 2).

We examined whether co-expression of EGFR with p185<sup>new</sup> and its derivatives could enhance transforming efficiency compared with cells expressing these mutant p185<sup>new</sup> proteins alone. Cell lines listed in Table I (except kinase-deficient T691stop and Er/T691stop clones) were able to form foci independent of ligand stimulation (data not shown). Co-expression of EGFR with p185<sup>new</sup> in Er/neu cells increased the ability to form foci, both in density and absolute number (by greater than 3-fold). However, co-expression of EGFR with kinase-active truncated mutant TAP<sub>stop</sub> or TΔ5 did not enhance focus formation efficiency in Er/TAP<sub>stop</sub> and Er/TΔ5 cells when compared with TAP<sub>stop</sub> and TΔ5 cells, respectively (data not shown).

The colony growth efficiency of these clones in soft agar is also summarized in Table I. B104-1-1 cells expressing high levels of p185<sup>new</sup> served as a positive control, while Er/T691stop clones served as a negative control and did not exhibit transformed colonies under the same conditions. Compared with B104-1-1 cells, cells expressing lower levels of oncogenic p185<sup>new</sup> formed colonies less efficiently. However, more colonies were observed in EGFR-co-expressing Er/neu cells. Co-expression of EGFR with p185<sup>new</sup> still permits functional heterodimerization in addition to homodimerization of either receptor, resulting in elevated biological activity, contributing to increased transforming activity in vitro. Cells expressing kinase-active truncated mutant TAP<sub>stop</sub> or TΔ5 mutant proteins alone displayed reduced colony growth efficiency in soft agar when compared with control B104-1-1 cells, although the expression levels of p185<sup>new</sup> variants in these cells were similar. Critically, co-expression of EGFR with TΔ5 or TAP<sub>stop</sub> did not increase colony growth efficiency in soft agar.

Tumorigenicity was studied by injection of these mutant clones individually into athymic mice. Results are presented in Table I, which summarizes receptor expression levels, tumor frequency, and tumor size. B104-1-1 cells expressing oncogenic p185<sup>new</sup> were used as a positive control and tumors caused by those cells appeared and grew quickly (with a latency of 5–7 days). No tumors were observed with kinase-deficient mutant clones T691stop and Er/T691stop cells (>10 weeks observation). Co-expression of EGFR and p185<sup>new</sup>, each at low levels, in Er/neu cells greatly accelerated tumor appearance (~2 weeks), and the tumors grew aggressively when compared with p185<sup>new</sup> cells that also expressed low level of oncogenic p185<sup>new</sup> (>4–5 weeks). Cooperative signaling between EGFR and p185<sup>new</sup> was thus also observed in tumorigenicity assays in vivo. TΔ5 protein expression was sufficient to induce tumors (latency period of 2–3 weeks), and TAP<sub>stop</sub> mutant receptor expression also resulted in tumor formation (latency of 4–5 weeks). Receptor expression levels for these two mutant proteins was close to that in B104-1-1 cells. Notably, co-expression of EGFR with these mutant proteins, i.e. in Er/TAP<sub>stop</sub> and in Er/TΔ5, did not promote tumor growth.

The failure of distinct endodomain interactions between p185<sup>new</sup> and EGFR, caused by an ectodomain deletion (TΔ5 mutant), or the lack of a functional COOH terminus (TAP<sub>stop</sub> mutant), clearly impairs signaling needed for transformation.

Structural Requirements of Trans-receptor Activation

**FIG. 5.** EGF-induced cell proliferation. Cells were plated in 96-well plates (3000/well) overnight in DMEM containing 5% FBS. After starvation in serum-free media for 48 h, cells were grown in the same media supplemented with various concentrations of EGF as indicated for an additional 48-h period. Cell proliferation was determined by the MTT assay as described under “Experimental Procedures.” The resultant OD<sub>600</sub> was plotted for an additional 48-h period. Cell prolif-
In contrast, the presence of T691stop in Er/T691stop cells suppressed the proliferative response to EGF, and cell growth was dramatically reduced (Fig. 5B). These results correlated with the inhibition of EGFR kinase (Figs. 3E and 4B). Interestingly, the EGFR in Er/TAPstop and Er/TΔ5 cells behaved normally in EGFR-dependent mitogenesis when compared with that in NE91 cells, except that the basal growth level was higher (Fig. 5, C and D) due to a more transformed phenotype (data not shown). These data correlated with previous observations (Figs. 2–4), suggesting that EGFR signaling is comparable in Er/TAPstop and in Er/TΔ5 clones to that seen in NE91 cells, i.e., neither enhanced nor suppressed. However, trans-receptor signaling was not observed due to either defective heterodimerization in Er/TAPstop cells or failure of heterodimerization in Er/TΔ5 cells.

**EGF-dependent MAP Kinase and PI 3-Kinase Activation**—To understand the mechanism underlying synergetic proliferative and transforming signal propagated by heteroreceptor interaction, we studied the EGF-induced MAP kinase and PI3 kinase pathways signaling phenomena. The proto-oncogenic p185 (Nneu) and its derivatives (NΔ5 or, N691stop) were co-expressed with EGFR, to evaluate EGF-dependent activation of downstream kinases, since p185 and TΔ5 are both constitutively active tyrosine kinases. An epitope-tagged HA-MAPK was also co-expressed with the combination of receptors in COS7 cells to examine downstream ERK activation.

Co-expression of p185, but not NΔ5, with EGFR increased MAP kinase activity upon EGF stimulation. In contrast, EGFR-mediated MAP kinase activity in N691stop coexpressing cells was suppressed when compared with cells expressing EGFR and an empty vector control (Fig. 6A). Equivalent protein expression levels of epitope-tagged HA-MAPK were developed by ECL. Lanes 1–8 in these panels are correspondent to those in panel A, D (lanes 7 and 8), cells were metabolically labeled with [35S]methionine and cell extracts were immunoprecipitated (IP) with 7.16.4 and analyzed in SDS-PAGE followed by autoradiography. Similar results were obtained in other two independent experiments.

FIG. 6. EGF-induced MAP kinase activity. COS7 cells transiently expressing exogenous HA-MAPK, EGFR, and wild-type or mutant p185 were treated with or without EGF (50 ng/ml) for 5 min as indicated. A, cells were then lysed, and anti-HA immunocomplexes were washed and underwent kinase reaction as described under “Experimental Procedures.” The phosphorylation level of myelin basic protein (MBP) was determined (Fig. 6A). Inter- nal signals were shown after autoradiography. B–D, equal amounts of cell extracts were used for examining ectopically expressed proteins. Antibodies used in immunoblot (IB) were indicated. Protein signals were developed by ECL. Lanes 1–8 in these panels are correspondent to those in panel A, D (lanes 7 and 8), cells were metabolically labeled with [35S]methionine and cell extracts were immunoprecipitated (IP) with 7.16.4 and analyzed in SDS-PAGE followed by autoradiography. Similar results were obtained in other two independent experiments.

FIG. 7. EGF-induced PI 3-kinase activity. COS7 cells transiently expressing EGFR and wild-type or mutant p185 were treated with or without EGF (50 ng/ml) for 5 min after serum starvation for 24 h. Equal amounts of cell extracts were immunoprecipitated by anti-Tyr(P) (4G10) and analyzed for PI 3-kinase activity as described under “Experimental Procedures.” Autoradiogram of thin layer chromatography plate exposed overnight is shown. The positions of origin (ori.), phosphatidylinositol 3-phosphate (PIP), and phosphatidylinositol 3,4-biphosphate (PIP2) were indicated by arrows. Data shown are representative of three individual experiments.

Activation of PI-3-kinase requires phosphorylation of the Src homology 2-containing adapter p85 by receptor tyrosine kinases. Phosphatidylinositols are critical signaling intermediates and influence cell growth, differentiation, and adhesion (52). ErbB family members, notably ErbB-3, have been shown to associate with the p85 subunit of PI 3-kinase (53). To examine the influence of wild-type or mutant p185 on EGF-dependent activation of PI 3-kinase, plasmids expressing EGFR with vector or p185 variants were transiently expressed in COS7 cells. PI 3-kinase activity was examined in serum-starved cells with or without EGF stimulation. We observed a similar magnitude of the EGF-induced PI 3-kinase activity in cells expressing EGFR only or Er/NΔ5. The PI 3-kinase activity was much greater in Er/p185 cells, and much weaker in Er/N691stop cells (Fig. 7). Expression patterns of these receptor proteins were determined (Fig. 6, C and D).

The observed super PI 3-kinase activity in Er/p185 cells may arise through the tyrosine phosphorylation of the p85 subunit by the heteroreceptor complexes. We believe heteroreceptor complexes are more active since truncated p185 proteins alone do not seem effective at interaction with p85 (data not shown). Induced PI 3-kinase and MAPK activities therefore paralleled the heterodimerization and trans-activation events depicted in Figs. 2–4, and biological results obtained in Table I and Fig. 5. Functional heterodimerization observed in Er/neu cells permits cooperation and diversification of signaling, which contrasts with the formation of signaling-defective com-
Functional heterodimerization requires the ectodomain for ligand-mediated physical associations, the endodomain for kinase transactivation, and the carboxyl terminus for cross-phosphorylation and combinatorial cellular signaling. Deletion of each subdomain results in inefficient heterodimerization, preventing kinase activation and defects in cooperative cellular signaling, respectively. *TD5, TΔ5*.

**DISCUSSION**

Using p185neu mutants, which retain the capacity to homodimerize, we observed that EGF-induced heterodimerization could occur. Heterodimerization was seen in cells co-expressing EGFR with TAPstop or T691stop mutant receptors, but not with the extracellular domain-deleted TΔ5 (Fig. 2), demonstrating that the ectodomain of p185*neu* is necessary and sufficient for heterodimerization with EGFR. Indeed, heterodimerization of the EGFR and N691stop form derived from proto-oncogenic p185*neu* has been observed to occur preferentially to either p185*neu* or EGFR-EGFR homodimerization (37).

Two alternative assays confirmed trans-activation of ErbB family proteins following heterodimer formation. Anti-phosphotyrosine blotting showed that enhancement of tyrosine phosphorylation in response to EGF occurred only in cells co-expressing EGFR with the full-length p185*neu* kinase, but not with the TAPstop or TΔ5 mutant receptors. It appears that EGFR and the T691stop neu mutant formed a kinase-inactive complex (Fig. 3), as described previously for the N691stop form derived from p185*neu* (22), but not with kinase-deficient p185*neu* (44), synergistically transformed rodent fibroblasts. EGFR and p185*neu* associates into an active kinase complex (24) which up-regulates EGFR receptor function by increasing EGF binding affinity, ligand-induced DNA synthesis, and cell proliferation (23). In the current studies, when EGFR was co-expressed with oncogenic p185*neu* at physiological levels (~10⁴ receptors/cell), we also observed enhancement of tumor growth (4-fold) *in vivo* and anchorage-independent growth (~2-fold) *in vitro*, compared with the cells expressing p185*neu* alone (Table I). Deletion of 122 amino acid residues from the carboxyl terminus of p185*neu* eliminates three known tyrosine autophosphorylation sites (TAPstop mutant), and causes impaired cellular signaling and transforming potency (41). Overexpression of EGFR with the carboxyl-terminally truncated TAPstop mutant receptor, although leading to an active heterodimeric complex, did not recover the diminished transforming potency of TAPstop (Table I), indicating that signaling propagation through the carboxyl terminus of p185*neu* could not be restored by association with full-length EGFR. These data emphasize that cooperative signaling requires not only the formation of an active kinase complex, but also a heteromeric functional carboxyl termini within the two receptor endodomains that recruit various downstream molecules required to generate signal to mediate cell growth and transformation.

The current results indicate that p185*neu*-EGFR heterodimerization is greatly favored, even in the presence of the neu transmembrane point mutation that facilitates p185*neu* homodimerization (12). Together with the observation that ErbB-2 is the preferred heterodimerization partner of all ErbB members (36), these studies emphasize that Neu/ErbB-2 may mediate signaling diversity through structural interactions governed by particular ectodomain sequences. For instance,
ErB-3 is a less active kinase than other ErB proteins (57), but serves as a binding site for Neu differentiating factor (28) and forms a potent heterodimer with ErB-2, consequently engaging various downstream substrates. Neu-ErbB-2 may not be required for ligand binding, but may reconstitute signaling by laterally engaging other ErB proteins in some preferred, but not well understood manner.

Kinase phosphorylation increases the affinity of binding of Src homology 2 and Src homology 3 domain-containing substrates, and initiates a variety of cascades. The binding of Grb2-Sos complexes to the active EGFR activates the Ras/Raf/MAP kinase cascade (58). Another downstream effector whose importance in cell signaling and, potentially, in tumorigenesis is becoming increasingly understood is PI 3-kinase (52). PI 3-kinase activation has also been shown to be essential for induction of DNA synthesis by EGF (59). Current studies have demonstrated that EGF-induced ErbB heterodimers activate both the ERK and PI 3-kinase pathways. Functional wild-type heterodimers, but not defective mutant heterodimers, efficiently induce both ERK and PI 3-kinase activities, which demonstrate that EGF-induced ErbB heterodimers activate induction of DNA synthesis by EGF (59).

As depicted in Fig. 8, these results further support the notion that cooperative signaling caused by p85<sup>neu</sup>-EGF receptor ensembles requires the ectodomain for ligand-mediated physical association, while the intracellular domain provides contacts for efficient intermolecular kinase activation. The phosphorylated carboxyl terminus is essential for recruiting particular cellular substrates required for signal diversification.

In particular, specific ectodomain associations may therefore underlie the combinatorial interactions within the ErBb family required for signal diversification. These properties may be features that are used by many receptor ensembles involved in enzymatic signaling in cells.

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