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PRINCIPAL INVESTIGATOR: Mark I. Greene, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

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Philadelphia, Pennsylvania 19104-3246

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The studies have begun to identify the specific roles of distinct tyrosines in a receptor oncoprotein that plays a role in breast cancer. By using a variety of mutant forms of p185neu we showed that in vitro and in vivo activity of mutants that have an intrakinase Y→F mutation is dramatically reduced. To date we have successfully analyzed the role of the intrakinase tyrosine at position 882 and determined it is critical for the transforming activity of p185. The studies have consistently revealed that, biochemically and biologically, the tyrosine at 882 is critical for the receptor's activity. At present we believe that the tyrosine 882 may influence substrate access to the active site of the kinase. These results may help in the development of specific kinase inhibitors that can be used to block the activity of p185 homomers or heteromers that are active in the development of breast neoplasia.

Our recommendations for future studies are to use this information in the structural design of erbB kinase inhibitors.

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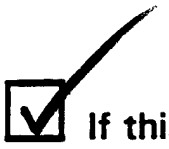

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Mark L. Gross 8/7/97

PI - Signature Date

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Introduction:

The p185 protein is a receptor which, when stimulated, conveys mitogenic signals to cells that can lead to their transformation. P185neu is often abnormally expressed in breast and ovarian cancers. In some adenocarcinomas it is not the overexpression of p185 that is thought responsible for transformation but rather its coexpression with the Epidermal Growth Factor receptor (EGFr). We had previously shown that p185 is able to form heteromeric assemblies with the EGFr and this assembly has far greater kinase activity than either homomer by itself. Many aspects of homomeric and heteromeric assembly function including internalization are determined by discrete regions of the endodomain.

The objective of these studies is to provide a better molecular understanding of the formation of homomeric and heteromeric complexes of the p185-EGFr family of erbB molecules. The scope of the project was to define regions of the endodomain of p185 that governed aspects of down-modulation and internalization of receptor complexes as well as separate regions that permitted the formation of active kinase complexes. The second aim was to begin to isolate and characterize ligand molecules that stimulate the p185 receptor.

Although no ligands that bind to p185 have been cloned we have isolated a protein termed Neu activating factor from a thymus derived transformed human cell line. One feature of the proposal was to clone the gene encoding this polypeptide and study its effect on transformation and cell growth of p185 expressing lines. These studies are underway; but, to date, we have no data to report. cDNA libraries are currently being screened with an ectodomain form of p185.

Mutation and overexpression of erbB family receptors has been found to lead to receptor activation(1) by inducing the formation of dimeric and oligomeric forms (2-3). The net effects of oligomerization are both enhanced kinase activity and subsequent signal transduction. Activation of erbB family receptors results in self-phosphorylation of receptors by an intermolecular mechanism and in the phosphorylation of substrates which are critical in initiating signal transduction events required for cell growth and differentiation. The interactions between receptor tyrosine kinases and specific signaling proteins are, in part, governed by phosphorylation at particular tyrosine residues in the carboxyl terminus of these receptors. Receptor autophosphorylation is, therefore, not only required for direct interaction and phosphorylation of protein substrates, but also is felt to be important in the regulation of the enzymatic tyrosine kinase activity.

In the case of the EGFr, self-phosphorylation of the cytoplasmic domain is known to occur at five tyrosine residues located in the carboxyl terminus outside of the enzymatic kinase domain (Y992, Y1068, Y1086, Y1148, and Y1173) (1-3). Many kinases are also activated by phosphorylation of a conserved Thr or Tyr site that lies within the catalytic core of the kinase domain. For example, a conserved tyrosine residue within the kinase domain of the v/c-src kinase has been located at position Y416. Autophosphorylation of this site is important for regulating kinase enzymatic function and biological activity of the src kinase (4). The corresponding tyrosine residue is important for regulating kinase function in many other receptor and cytoplasmic tyrosine kinases, including v-fps (5), the insulin receptor (6), the colony

stimulating factor I-receptor (CSF-IR) (7), and the platelet-derived growth factor receptor (PDGFR) (8). However, the analogous site within the catalytic core of the EGFr kinase domain, Y845, has been reported to be less relevant for tyrosine autophosphorylation of the mature 170kDa EGFr, for EGF-induced stimulation of DNA synthesis, or for EGF-dependent transformation of NIH3T3 cells (9). Nevertheless, mutations in charged residues adjacent to Y845 decrease the Vmax for receptor self-phosphorylation, suggesting that this surface region stabilizes the catalytic core of the kinase domain and is important for maximal self-phosphorylation activity of the kinase domain (9).

Body:

The general assumptions of the initial study were that by creating a series of endodomain mutants followed by transfection into fibroblast lines we could deduce the role of certain critical tyrosine residues.

Methods

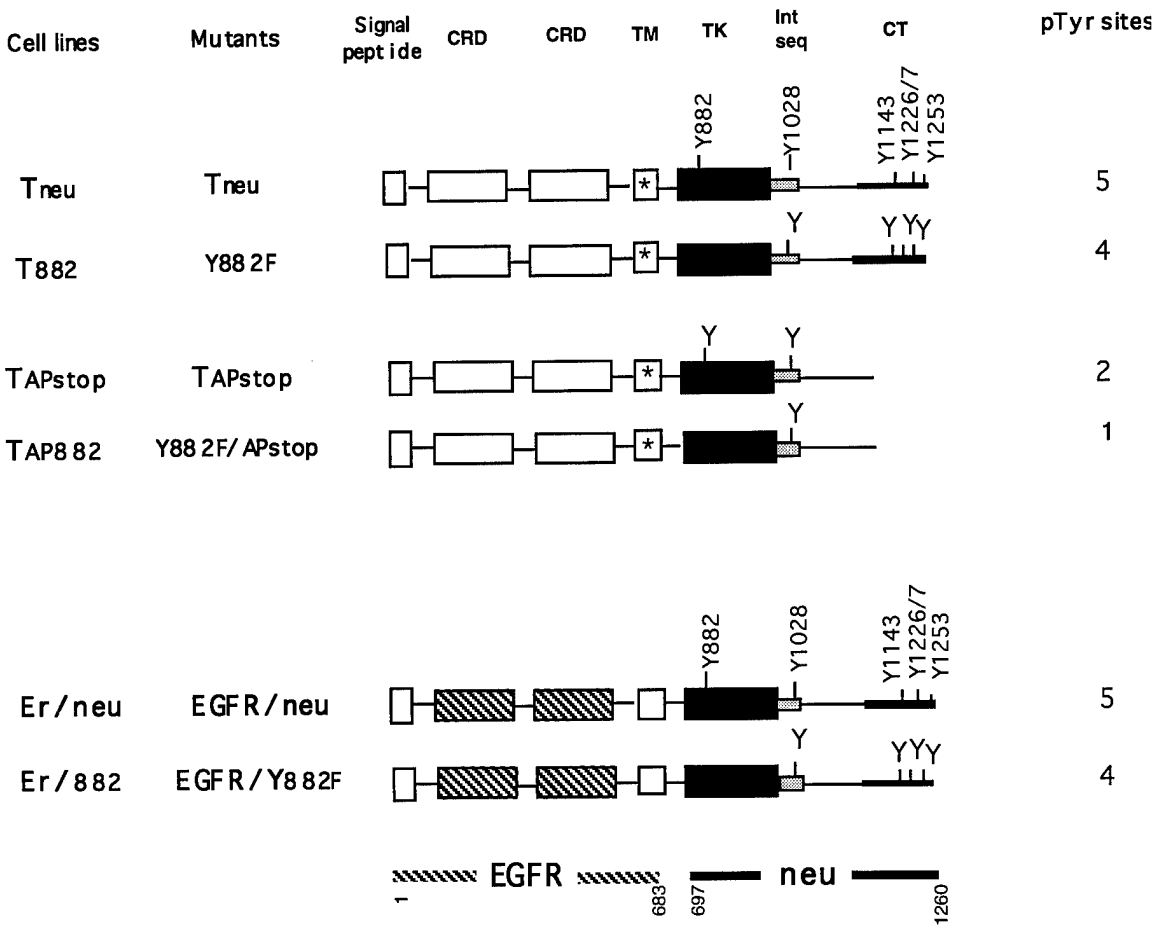
Construction of Mutants and Expression Vectors

All the following mutant p185neu cDNAs were derived from rat oncogenic neu cDNA (Tneu) containing a single mutation (V664E) in the transmembrane region. Briefly, the mutant Y882F was derived from Tneu by site-directed mutagenesis by substituting a phenylalanine for Tyrosine-882. The TAP/stop mutant was also made by site-directed mutagenesis by introducing a stop codon which was 120 amino acids from the carboxyl terminus. The T882F/APstop mutant was made by a subcloning strategy to generate both the Y882F substitution and carboxyl terminal deletion. To make a chimeric mutant of EGFr and TneuY882F, a 6.7 kb SalI-XbaI fragment from an EGFr-neu expression vector, pSV2EGFr/neu, was ligated to a 2.3 kb fragment containing the 3' region of the Y882F mutant. The chimeric receptors have the ectodomain of the EGFr and an endodomain derived from a form of neu. All the mutant constructs were verified by DNA sequencing (2,3,10-13).

Derivation of Stably Transfected Cell Lines

The wild type or mutant *neu* cDNAs were subcloned into the pMuLVLTRneo^r expression vector and then transfected into NR6 fibroblasts lacking endogenous EGFr's with neomycin selection to generate mutant neu-expressing subclones. The expression of mutant neu proteins in resultant colonies was confirmed by flow cytometry using an anti-p185neu monoclonal antibody (MAb) 7.16.4 The resultant transfected cell lines were designated Tneu, T882, TAP/stop, TAP882, Er/neu, and Er/882. Using flow cytometric analysis, it was determined that neu-derived proteins were expressed at comparable levels in Tneu and T882 cells, and in TAP/stop and TAP882 cells, although the latter two cell lines had higher surface expression levels than the Tneu and T882 cell lines. These transfected clones were maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS, Hyclone).

Mutant Cell Lines



Analysis of transforming characteristics

Anchorage-independent growth was determined by assessing the colony forming efficiency of cells suspended in soft agar. Cells (250-1000) were suspended in 0.18% agarose/5% FBS-DMEM and plated on 0.25% of basal agar in 60 mm tissue culture plates. 0.5 ml of DMEM medium (5% FBS, 20 mM Hepes, pH 7.5) was added to soft-agar cultures once a week for the duration of the experiment. Colonies (>0.3 mm) were visualized on day 21 for all cell lines after staining with *p*-iodonitrotetrazolium violet (1 mg/ml). Each cell line was examined in triplicate for three separate experiments. Number of colonies reported represented the mean of triplicate samples (11-13).

To analyze tumor growth in athymic mice, cells (0.5×10^6) of each line were suspended in 0.1 ml of PBS and injected intradermally in the mid-dorsum of NCR nude mice. PBS alone was also injected as a control. Animals used in this study were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania. Tumor growth was closely monitored for up to 8 weeks (2,11-13).

Antibodies

The monoclonal antibody (MAb) 7.16.4 against the ectodomain of p185neu was produced from hybridoma cells as described previously (2-3). The polyclonal rabbit antiserum reactive with the neu intracellular domain, designated anti-Bacneu, was also utilized. A monoclonal anti-phosphotyrosine antibody (pY20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

In Vitro Kinase Assay

Cells were plated in 100 mm culture dishes and the next day were washed twice in ice cold PBS and lysed in 1 ml of lysis buffer (50 mM Hepes, pH 7.5, 150mM NaCl, 3% Brij-35, 2 mM EDTA, 0.02mg/ml Aprotinin, 10% glycerol, 1.5 mM $MgCl_2$). Cell lysates were centrifuged at 20,000 g for 15 min. Protein concentrations of cell lysate were measured with the D_C Protein Assay (Bio-Rad). Lysates that contained comparable amounts of neu-derived proteins (as determined by Western blotting) were used for immunoprecipitation with the anti-neu MAb, 7.16.4. 40 microliters of 50% (vol/vol) protein A-sepharose were used to collect the immune complexes, which were then washed three times with wash buffer (50 mM Hepes, 150 mM NaCl, 0.1% Brij-35, 2 mM EDTA, 0.01 mg/ml Aprotinin, 0.03 mM Na_3VO_4). The pellets were suspended in 20 microliters of 20 mM HEPES (pH 7.4, 5 mM $MnCl_2$, 0.1% Brij-35, 0.03 mM Na_3VO_4, 0.02 mg/ml Aprotinin) containing 5 uCi of ^{32}P-γ-ATP, and incubated at room temperature for 30 min. The reaction were terminated by the addition of 3x electrophoresis sample buffer containing 2 mM ATP. After incubation at 100°C for 3 min, samples were then analyzed by SDS-PAGE (11-13).

MTT(3,(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide) Assay of Cell Proliferation

The MTT assay for measuring cell growth has been reported previously (2-3). Briefly, cells (5000) of each cell line were seeded in 96-well plates overnight in DMEM containing 5% FBS. Cells were starved in ITS-DMEM for 48 hours, then cultured in 100 microliters of the same medium plus various concentrations of EGF for 48 hours. 25 microliters of MTT solution (5 mg/ml in PBS) were added to each well, and after 2 hours of incubation at 37°C, 100 microliters of the extraction buffer (20% w/v of 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 ELISA reader. Each value represented a mean of four samples.

Research findings

All mutant neu constructs were derived from Tneu and therefore contain the activating transmembrane point mutation. Site-directed mutagenesis was used on the Tneu construct to generate a phenylalanine (F) at position 882 instead of a tyrosine (Y). Tneu and Y882F were used to generate an additional set of mutants, TAPstop and Y882F/APstop, respectively, by deleting 120 amino acids from the carboxyl terminus. Additionally, a mutant chimera, EGFr/Y882F, was generated from the EGFr/neu chimeric receptor. These mutant proteins were expressed in

NR6 cells, a fibroblast cell line which lacks endogenous EGF receptors. Tneu refers to a murine fibroblast cell line which expresses oncogenic p185neu.

Tyrosine phosphorylation

Immunoprecipitation of mutant p185neu forms followed by immunoblotting with an anti-phosphotyrosine antibody was performed in order to assess whether substitution of tyrosine-882 was associated with a reduction in total phosphotyrosine content of oncogenic p185neu immunocomplexes. Cells expressing Y882F (T882) appeared to have comparable levels of full-length tyrosine phosphorylated p185neu relative to total receptor, which was also observed in a comparison between mutants TAPstop (TAPstop cells) and Y882F/APstop (TAP882 cells) In cells expressing full-length p185neu mutants (Tneu and T882 lines), an additional faster migrating form of the protein was observed which presumably represents a precursor form of p185neu. Our results indicate that this precursor form of p185neu is also differentially phosphorylated on tyrosine residues. Deletion of the carboxyl-terminal 120 amino acids produced a protein of approximately 170-175kDa and resulted in the detection of only one form of mutant p185neu receptor.

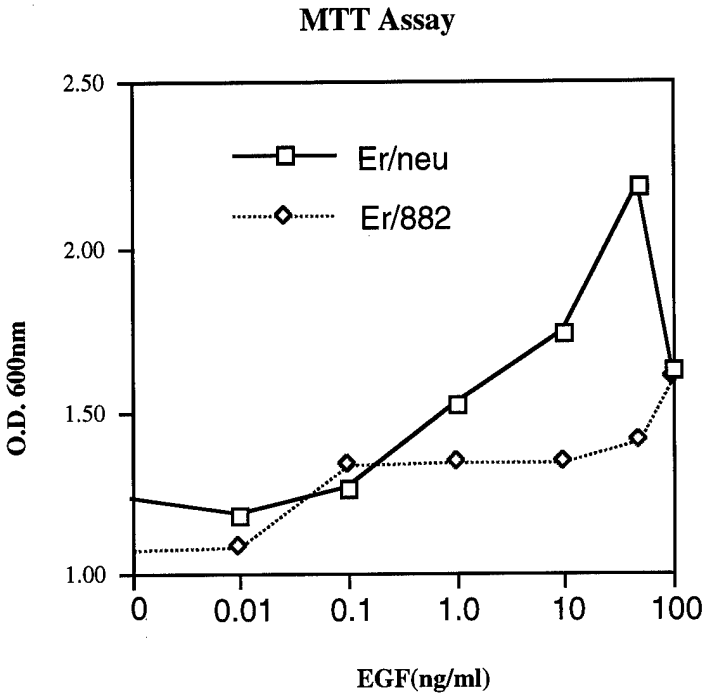
Effect of Y882F substitution on in vitro kinase activity of p185neu

Anti-neu immunocomplexes from cell lines expressing p185neu-derived mutant proteins were suspended in kinase reaction buffer containing [^{32}P]-γ-ATP and protein samples were then separated by 8% SDS-PAGE. Y882F mutant neu proteins expressed in T882 cells have a dramatically reduced total phosphorylation content in this assay compared to oncogenic p185neu. Additionally, deletion of the carboxyl terminal 120 amino acids caused a similar reduction of total phosphotyrosine content of mutant neu proteins of 170-175kDa, suggesting that the reduced autophosphorylation of Y882F could not be explained by decreased autophosphorylation of the three terminal autophosphorylation sites. In addition to decreased autophosphorylation of p185neu receptors, substitution of tyrosine-882 resulted in decreased phosphorylation of exogenous substrates. Differences were more pronounced between cells expressing Tneu and Y882F than between cells expressing TAPstop and Y882F/APstop. This result suggests that the conformational changes resulting from the deletion of the carboxyl terminal 120 amino acids influences substrate accessibility to the activation loop of the p185neu kinase domain, although this may not be generalizable to all biological substrates. Decreased tyrosine in vitro kinase activity of p185neu mutant receptor proteins was consistently observed in lysates derived from various Y882F mutant cell lines (T882, TAP882).

Y882F substitution inhibits EGF-induced cell proliferation of cell lines expressing EGFr/p185neu chimeric receptors

To analyze mitogenic signaling in response to ligand, the intracellular domains of p185neu and Y882F were independently fused with the extracellular domain of the EGF receptor. Using the MTT assay, we determined that the proliferation of cell lines expressing these chimeric receptors was EGF-dependent. We had previously shown that the EGF dose needed for a proliferative response to

ligand-induced DNA synthesis or cell proliferation was in the ng/ml range in fibroblasts and human transformed cell lines expressing the EGFr only. Cell growth 48h after EGF stimulation in Er/882 cells expressing EGFr/Y882F chimeric receptors was less than in those expressing EGFr/neu receptors (Er/neu cell line).



MTT assay: Response of different cell lines to EGF treatment. On day 1, individual 96-well plates were seeded with 5000 cells/well of cells expressing EGFR/neu or EGFR/Y882F mutant receptors in 5% FBS medium and incubated overnight at 37°C. On day 2, the medium was changed to serum-free medium with different concentration of EGF and the microplates were incubated at 37°C for 48 hours. On day 4, MTT was added (1 mg/ml) and after 4 hours incubation at 37°C, cells were lysed by buffer containing SDS and DMSO at pH4.7. The OD was then measured on day 5 at 600nm using an ELISA reader.

Substitution of Y882 inhibits transformation of cell lines expressing oncogenic p185neu and EGFr/neu chimeric receptors as determined by anchorage-independent cell growth

The ability of murine fibroblasts to form colonies in soft agar was addressed in order to determine whether tyrosine-882 was relevant to the transforming activity of p185neu. Cells expressing Y882F were inhibited in their transforming efficiency in vitro. Elimination of the three carboxyl terminal autophosphorylation sites (TAPstop) resulted in reduced oncogenicity relative to full-length oncogenic Tneu which was further reduced by substituting tyrosine-882 with phenylalanine (Y882F/APstop). In order to assess the effect of tyrosine-882 substitution on ligand-dependent transformation, EGFr/neu chimeric proteins were expressed in murine fibroblasts. The Y882F neu intracellular domain was fused to the extracellular domain of the EGF receptor. When these cells were examined for their growth

capability, colony growth in soft agar of EGF-dependent fibroblasts was inhibited as a consequence of possessing the Y882F substitution in the p185neu intracellular region.

In vivo tumorigenicity

A comparison of tumor growth in athymic mice was then performed with the Y882F mutant cell lines. Oncogenic p185neu-expressing fibroblasts (Tneu) form rapidly growing tumors when implanted subcutaneously in nude mice. Full-length Y882F mutant receptors are inhibited in their ability to mediate transformation in vivo. Replacement of the carboxyl terminal three autophosphorylation sites in p185neu proteins containing the activating transmembrane point mutation (TAPstop) also resulted in a significant diminution of tumor forming efficiency. Visible tumor growth did not occur in these animals until four weeks, which was beyond the time point that animals implanted with Tneu cells were sacrificed from tumor burden. Again, substitution of tyrosine-882 was associated with further inhibition of transforming efficiency in vivo.

Discussion

The studies have begun to identify the specific roles of distinct tyrosines in a receptor oncoprotein that plays a role in breast cancer. By using a variety of mutant forms of p185neu we showed that in vitro and in vivo activity of mutants that have an intrakinase Y→F mutation is dramatically reduced. The studies have consistently revealed that, biochemically and biologically, the tyrosine at 882 is critical for the receptor's activity. At present we believe that the tyrosine 882 may influence substrate access to the active site of the kinase.

Our recommendations for future studies are to use this information in the structural design of erbB kinase inhibitors.

Conclusions:

To date we have successfully analyzed the role of the intrakinase tyrosine at position 882 and determined it is critical for the transforming activity of p185. These results may help in the development of specific kinase inhibitors that can be used to block the activity of p185 homomers or heteromers that are active in the development of breast neoplasia.

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APPENDIX

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Supporting and background publications:

Dougall, W.C., Qian, X., Miller, M., and M.I. Greene. (1996). *DNA and Cell Biology* **15**, 31-40.

Murali, R., Brennan, P.J., Kieber-Emmons, T., and M.I. Greene. (1996). *Proc. Natl. Acad. Sci.* **93**, 652-657.

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Association of Signaling Proteins with a Nonmitogenic Heterodimeric Complex Composed of Epidermal Growth Factor Receptor and Kinase-Inactive p185$^{c\text{-}neu}$

WILLIAM C. DOUGALL,[1] XIAOLAN QIAN, MARSHA J. MILLER, and MARK I. GREENE

ABSTRACT

The functional consequences of heterodimer formation between the epidermal growth factor receptor (EGFr) and the p185$^{c\text{-}neu}$ receptor tyrosine kinase include increased mitogenic and transformation potencies. To determine the possible alteration of signal transduction pathways resulting from this heteromeric complex, the capacity of several signaling proteins to associate with the heterodimeric receptors has been assayed. The *in vivo* interaction with the EGFr/p185$^{c\text{-}neu}$ heterodimer of several signal transduction proteins, including phospholipase C-γ1 (PLC-γ1), the p85 subunit of phosphotidylinositol 3-kinase, the *ras* GTPase activating protein, SHC, NCK, p72RAF, and the tyrosine phosphatase SHPTP2, was measured by coimmunoprecipitation. The binding of these signaling proteins to a complex composed of EGFr and a kinase-inactive form of p185 (p185K757M) was not impaired, even though the mitogenic and transformation activity of this complex had been abrogated. In addition, the EGF-induced phosphorylation of GAP, p85, and PLC-γ1 did not correlate with the dominant-negative action of p185K757M on EGFr function. Thus, substrate association and phosphorylation do not correlate stringently with the mitogenic and transforming activity of this receptor complex, suggesting additional pathways or mechanisms vital to EGFr/p185$^{c\text{-}neu}$ heterodimeric signaling.

INTRODUCTION

RECEPTOR TYROSINE KINASES transmit signals resulting from polypeptide ligand binding by the phosphorylation and activation of cellular proteins. Two prototypic type I transmembrane receptors, the epidermal growth factor receptor (EGFr) and the product of the rat c-*neu* or the human c-*erbB-2* gene (p185$^{c\text{-}neu/c\text{-}erbB\text{-}2}$) affect signal transduction mechanisms by ligand-induced receptor oligomerization, which results in the activation of the intrinsic tyrosine kinase and receptor autophosphorylation (Weiner *et al.*, 1989a,b; Ullrich and Schlessinger, 1990). This ligand-induced mechanism has been deregulated in the oncogenic p185^{neu} protein as a result of a point mutation in the transmembrane region leading to constitutive oligomerization, enzymatic activation, and cellular transformation (Bargmann and Weinberg, 1988; Weiner *et al.*, 1989a,b).

The overexpression of the human c-*erbB-2* protein has been observed in a large number of human adenocarcinomas (including breast, ovarian, stomach, pancreatic, and bladder) and correlates with a poor clinical prognosis (Slamon *et al.*, 1987). The hypothesis that human c-*erbB-2* gene amplification and protein overexpression causes cellular transformation by receptor oligomerization and subsequent tyrosine kinase activation in a ligand-independent manner has been confirmed experimentally (Di Fiore *et al.*, 1987; Chazin *et al.*, 1992; LeVea *et al.*, 1993; Samanta *et al.*, 1994).

A third mechanism for cellular transformation by type I growth factor receptors results from the hetero-oligomeric interaction of two different receptor proteins (EGFr and p185$^{c\text{-}neu}$). Expression of moderately high levels of EGFr and p185 proteins together in rat fibroblasts led to enhanced activation of the receptor tyrosine kinase complex (Qian *et al.*, 1992), enhanced proliferative response to EGF (Wada *et al.*, 1990), and transformation of fibroblasts in the absence of exogenously added growth factor (Kokai *et al.*, 1989). The critical role for EGFr/p185 heteroreceptor interactions in signal transduction has been shown in two ways: (i) the inhibition of transformation by down-modulation of either receptor protein by monoclonal antibodies (Wada *et al.*, 1989); (ii) the loss of EGF-induced responses in cells that express kinase-

Center for Receptor Biology and Division of Immunology, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.
[1]Present address: Department of Molecular Biology, Immunex Corporation, 51 University St., Seattle, WA 98101.

deficient forms of p185 (Qian *et al.*, 1994a,b). The regulation of receptor kinase function by hetero-receptor interactions has been suggested to be a general mechanism by which signal transduction pathways are modulated in different differentiation, growth, and transformation circumstances (for review, see Dougall *et al.*, 1994; Lemmon and Schlessinger, 1994; Heldin, 1995).

Receptor tyrosine kinase signaling is initially mediated by a number of tyrosine kinase substrates containing Src homology 2 (SH2) domains including phospholipase C-γ (PLC-γ), phosphatidylinositol-3 kinase, GTPase activating protein, SHPTP-2 tyrosine phosphatase, and the JAK/STAT proteins (van der Geer *et al.*, 1994; Marshall, 1995). In addition, the activation of the *ras* pathway by receptor tyrosine kinases leads to the regulation of serine/threonine protein cascades including Raf and the MAP kinase (MAPK) pathway (for review, see Johnson and Vallaincourt, 1994). The specificity of the growth factor signaling can thus be mediated at several levels: the initial ligand signal; the relative expression and hetero-interaction of growth factor receptors; the relative strength or duration of substrate activation; and/or the availability of cellular substrates responding to this signal.

A central question in attempting to understand how hetero-receptors (such as the EGFr and p185 tyrosine kinases) mediate enhanced and/or altered signaling pathways is to determine which cellular substrates couple with the receptor complex. Recent evidence has suggested the importance of cross-phosphorylation of associated receptor proteins as a mechanism for enhanced signal transduction (Soltoff *et al.*, 1994; Qian *et al.*, 1995). The dominant negative effect of a kinase-inactive $p185^{c\text{-}neu}$ mutant protein on EGFr function suggests that normal signal pathways have been disrupted (Qian *et al.*, 1994a,b). In the present study we have utilized the interaction of the kinase-inactive p185 protein (p185K757M) with the wild-type EGFr as a model system to determine the efficiency of substrate association with the heterodimeric complex. These results indicate that although EGF-induced functions (including mitogenesis) were abrogated in these cells, the association and phosphorylation of several cellular signaling molecules with the p185/EGFr heterodimer still operates efficiently in an EGF-dependent fashion.

MATERIALS AND METHODS

Cell-lines and antibodies

NR6 cells (Pruss and Herschman, 1977) were devoid of endogenous EGFr and $p185^{c\text{-}neu}$ expression and were used as the parental cell line for further transfection. M1 cells express the wild-type human EGFr and wild-type rat $p185^{c\text{-}neu}$ (Kokai *et al.*, 1989). NEN757 cells express wild-type human EGFr and a kinase inactive form of rat $p185^{c\text{-}neu}$ (p185K757M) in which the lysine at position 757 was altered to a methionine. All cell lines utilized in this study were characterized periodically for correct expression of receptors by flow cytometry and immunoprecipitation/Western blotting.

Monoclonal antibody (mAb) 7.16.4 against the extracellular domain of rat $p185^{c\text{-}neu}$ was produced from hybridoma cells as described previously (Drebin *et al.*, 1984) and did not cross-react with EGFr or other members of the EGFr family (Dougall and Qian, unpublished). Anti-p185 carboxyl-terminal antiserum NCT (LeVea *et al.*, 1993) was specific for p185; anti-EGFr antisera CT was specific for EGFr (kindly provided by Stuart Decker); anti-Bacneu antiserum against rat $p185^{c\text{-}neu}$ intracellular domain (Myers *et al.*, 1992) recognized both p185 and EGFr; anti-phosphotyrosine monoclonal antibody 4G10, and polyclonal antisera against PLC-g, SHPTP2, GAP, and p85 (PI3-kinase) were purchased from UBI (Lake Placid, NY). Polyclonal anti-Shc antisera was purchased from Transduction Laboratories (Lexington, KY). Each of these antibodies were tested for cross-reactivity to baculovirus-expressed rat p185 protein.

EGF treatment of cells

Cells were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin. Cells were plated at a density of 1×10^6/10-cm dish and cultured for 2 days; the media was decanted, cells were washed with PBS and media minus serum for 16 hr. Murine EGF (GIBCO/BRL) was added to the various concentrations indicated.

Immunoprecipitation and Western blotting

Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed with 1 ml of PLCLB (PLC lysis buffer: 50 m*M* HEPES pH 7.5, 150 m*M* NaCl, 10% glycerol, 1% Triton X-100, 1 m*M* EGTA, 1.5 m*M* $MgCl_2$, 100 m*M* NaF, 10 m*M* sodium pyrophosphate) supplemented with 2 m*M* sodium orthovanadate, 1 m*M* phenylmethylsufonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Lysates were clarified by centrifugation at $14{,}000 \times g$ for 15 min at 4°C and supernatants were immunoprecipitated with the various antibodies for 1 hr at 0°C. Immune complexes were purified with Protein A–Sepharose and washed twice with HNTG buffer (20 m*M* HEPES pH 7.5, 150 m*M* NaCl, 10% glycerol, 0.1% Triton X-100, 1 m*M* sodium orthovanadate, and protease inhibitors as described above), once with HNTG buffer containing 500 m*M* NaCl, and finally with HNTG buffer containing 150 m*M* NaCl. Controls demonstrated that the antibodies/antisera used were not limiting. Samples were boiled for 5 min in $NaDodSO_4$ sample buffer prior to $NaDodSO_4$-PAGE. Western blotting was performed as described previously (Wada *et al.*, 1990) and developed with anti-mouse or anti-rabbit coupled to horseradish peroxidase (Boehringer Mannheim) using enhanced chemiluminescence (ECL, Amersham). The intensity of the bands was analyzed using a Phosphorimager (Molecular Dynamics, Inc.). To determine the amount of coimmunoprecipitated receptor proteins proportional to the total receptor protein content, both receptors were simultaneously immunoprecipitated with specific antisera and serial dilutions of the immune complexes were blotted with anti-receptor antibodies.

*Expression and purification of glutathione-S-transferase fusion protein/*in vitro *SH2 binding assays*

The plasmid encoding the recombinant glutathione-*S*-transferase/p85 SH2 fusion protein (GST-p85SH2) was provided by

Dr. Ed Wood (Burroughs Wellcome Co.). This fusion protein was expressed using the pGEX2T vector and has been described previously (Yamamoto, 1992). It encodes amino acids 330–724 of the human p85 protein and includes both SH2 domains. The glutathione-*S*-transferase fusion protein was purified by glutathione affinity chromatography according to published procedures (Yamamoto *et al.*, 1992). The proteins were analyzed by NaDodSO$_4$-PAGE and a single band at the predicted molecular weight (70 kD) was identified. Protein concentration was determined using the Bradford assay.

Binding of GST fusion proteins to cellular proteins was performed in 1% Triton X-100 lysates (PLCLB). Five micrograms of the GST fusion protein was mixed with total cellular lysates at 4°C for 1 hr. Protein complexes were recovered by adding 40 μl of glutathione-agarose (Pharmacia) and continued mixing by rotation at 4°C for 30 min. The complexes were washed as described above. These data indicate that the p85 subunit of PI3-kinase associates (by virtue of its SH2 domains) with phosphorylated EGFr and p185$^{c\text{-}neu}$ after EGF treatment.

RESULTS

EGF-induced tyrosine phosphorylation

Alteration of the ATP binding site of p185 at position 757 from a lysine to methionine (K757M) results in the inactivation of the tyrosine kinase activity and the loss of tyrosine autophosphorylation (Weiner *et al.*, 1989; Qian *et al.*, 1994a). Recently, we have shown that stable co-expression of this kinase-inactive p185 (p185K757M) with the wild-type EGFr has profound effects on various EGF-induced functions, including receptor down-modulation and degradation, DNA synthesis, EGFr affinity for EGF, and cellular transformation (Qian *et al.*, 1994b). Both p185K757M and the 170-kD EGFr protein become rapidly phosphorylated on tyrosine residues following EGF treatment as shown by anti-phosphotyrosine immunoprecipitation followed by Western blotting with anti-receptor antibodies (Fig. 1, lanes 1, 2, 5, and 6). Approximately the same percentage (50%) of the cellular pool of EGFr and p185K757M become phosphorylated in NEN757 cells as in M1 cells co-expressing EGFr and wild-type p185 (Qian *et al.*, 1992). These data confirm that transphosphorylation of the kinase-inactive p185K757M by EGFr and autophosphorylation of EGFr were not significantly impaired in these cells. Immunoprecipitation with receptor-specific antisera demonstrated that the total levels of protein did not change during EGF treatment (Fig. 1, lanes 3, 4, 7, and 8), although the mobility of EGFr and p185 was reduced due to the increased phosphorylation.

Co-expression of the p185K757M protein with EGFr (in the NEN757 cell line) reduces the percentage of high-affinity (K_d = 1.3–7.5 × 10^{-11} *M*) EGF receptors seen in cells expressing only EGFr (NE91 cells) or cells expressing both wild-type p185$^{c\text{-}neu}$ and EGFr (M1 or NENB2 cells) from 5% to 0.5%, while the low-affinity (k_d = 5 × 10^{-9} *M*) EGF-binding subclass was unaltered (Qian *et al.*, 1994b). Accordingly, it was necessary to demonstrate the relative efficiency of total tyrosine phosphorylation in NEN757 cells relative to the other cell lines. Figure 2 illustrates an EGF dose–response curve of total cellular tyrosine phosphorylation in the cell lines M1 and

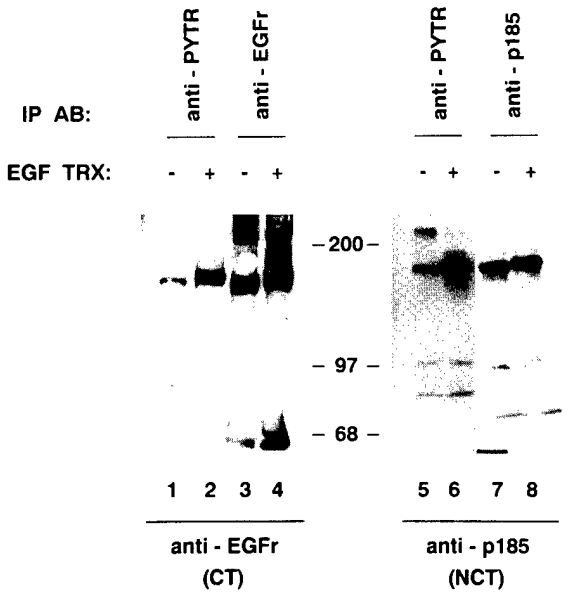

FIG. 1. Tyrosine phosphorylation of EGFr and p185$^{c\text{-}neu}$ receptor tyrosine kinases in NEN757 cells. Serum-starved cells were treated with 100 ng/ml of EGF for 15 min at 37°C before lysis with 1% Triton X-100-containing buffer. Immunoprecipitated proteins were separated by NaDodSO$_4$-PAGE (8% gel) and analyzed by anti-receptor or anti-PTYR antibodies. EGF-dependent tyrosine phosphorylation of the EGFr was demonstrated by anti-PTYR precipitation followed by immunoblotting with specific anti-sera (CT) against p170 EGFr (lanes 1 and 2). EGFr was directly immunoprecipitated with anti-EGFr antisera and immunoblotting with anti-EGFr antibodies (lanes 3 and 4). EGF-dependent tyrosine phosphorylation of the p185K757M was demonstrated by anti-PTYR precipitation followed by immunoblotting with specific anti-sera against p185$^{c\text{-}neu}$ (NCT) (lanes 5 and 6). Lanes 7 and 8 indicate direct immunoprecipitation of p185K757M followed by immunoblotting with NCT anti-sera.

NEN757. Both cell lines appear to respond efficiently to EGF in terms of tyrosine phosphorylation, and optimal tyrosine phosphorylation was observed at EGF concentrations of 50–100 ng/ml. Both cell lines also responded similarly over a defined time course to EGF treatment (unpublished data). The presence of the high-affinity EGFr in M1 cells does not appear to affect significantly the pattern of proteins phosphorylated after EGF treatment. Differences in phosphotyrosine-containing proteins were mostly quantitative, although some qualitative differences were also observed between the two cell lines upon longer exposures of the anti-phosphotyrosine immunoblots (Fig. 3). In each subsequent experiment, cells were treated with 100 ng/ml (16 n*M*) of EGF for 15 min unless indicated otherwise.

Shc, Grb2, and p72Raf proteins are bound by the phosphorylated EGFr/p185$^{c\text{-}neu}$ heterodimeric complex

The total level of tyrosine phosphorylation observed in the NEN757 cell line indicated that EGF-inducible kinase activity was still operable, which is in agreement with our previous re-

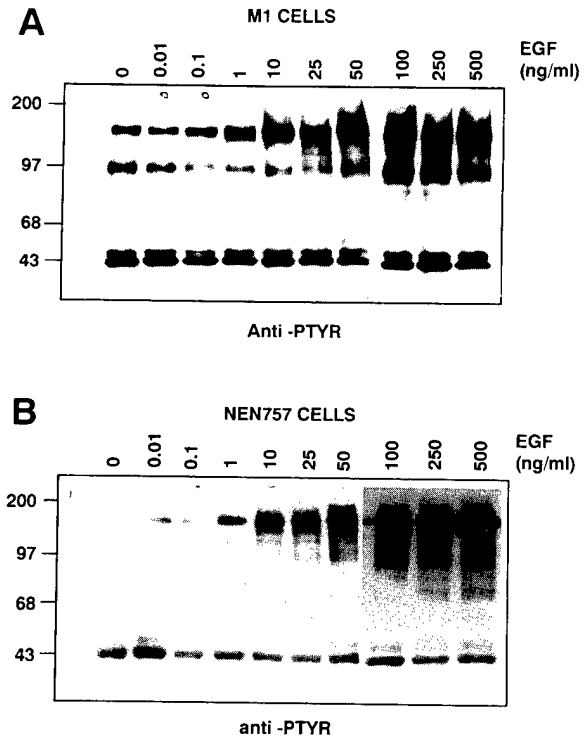

FIG. 2. Tyrosine phosphorylation of cellular proteins as a function of EGF dosage. Serum-starved cells were treated with indicated concentrations of EGF for 15 min at 37°C before lysis with 1% Triton X-100-containing buffer. Phosphotyrosine-containing proteins were precipitated with anti-PTYR antibodies, separated by $NaDodSO_4$-PAGE (8% gel), and analyzed by anti-PTyr immunoblotting. Molecular weight markers are indicated at left of each panel. The prominent band at 55 kD is the Ig heavy chain. A. M1 cells that express both wild-type rat $p185^{c\text{-}neu}$ and human EGFr proteins (Kokai *et al.*, 1989). B. NEN757 cells that express wild-type EGFr and a mutant form of $p185^{c\text{-}neu}$ (p185K757M), which lacks functional kinase activity.

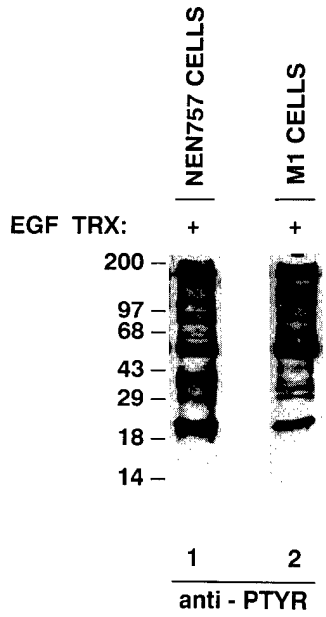

FIG. 3. Tyrosine phosphorylation substrates of the active (M1 cells) and inactive (NEN757 cells) EGFr/p185 heterodimeric complex. Cell lines were treated with EGF (100 ng/ml) for 15 min and tyrosine phosphorylated proteins were identified by anti-phosphotyrosine immunoprecipitation and anti-phosphotyrosine immunoblotting. Proteins were resolved on a gradient 4–20% $NaDodSO_4$-PAGE.

port (Qian *et al.*, 1994a) and indicates that the loss of EGF-induced mitogenesis in these cells was not due to a loss of kinase activity or protein phosphorylation. To examine whether effector/substrate proteins were coupled to the EGFr and p185K757M heterodimer, co-precipitation experiments were performed with anti-substrate antibodies. We initially examined three proteins implicated in the *ras* signaling pathway and subsequent activation of the MAP kinase system: Grb2, Shc, and p72Raf.

Lysates from NEN757 cells that were either treated with EGF or untreated were subjected to immunoprecipitation with antibodies specific for Grb2, SHC, and p72Raf. After resolution of proteins by $NaDodSO_4$-PAGE, samples were immunoblotted with antisera specific for either phosphotyrosine (clone 4G10), EGFr (anti-CT sera), or $p185^{c\text{-}neu}$ (anti-NCT sera). Figure 4 illustrates that a tyrosine-phosphorylated protein complex of approximately 170–190 kD is coimmunoprecipitated with each of these substrate/effector proteins after EGF treatment. Blotting with specific anti-receptor antisera confirmed that both EGFr

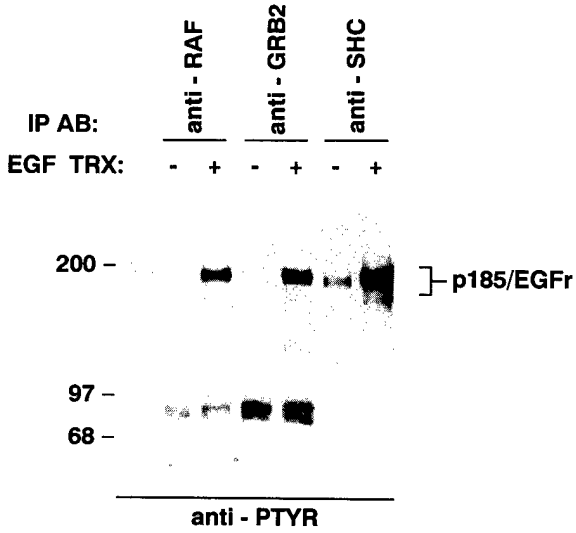

FIG. 4. EGF-induced complex formation of EGFr and $p185^{c\text{-}neu}$ proteins with RAF, GRB2, and SHC. NEN757 cells were treated with 100 ng/ml of EGF for 15 min as indicated. Total cellular lysates were immunoprecipitated (as described in Materials and Methods) with polyclonal antisera against the human RAF or SHC proteins or monoclonal antibodies reactive with the rat GRB2 protein. Proteins were fractionated on $NaDodSO_4$-PAGE (8%) and immunoblotted with antibodies against phosphotyrosine. The tyrosine-phosphorylated EGFr/p185 heterodimer was visualized at 170–190 kD. The sizes of marker proteins are indicated in kilodaltons.

and p185$^{c\text{-}neu}$ were present in this complex (data not shown and Qian *et al.*, 1994a). The size heterogeneity of the p185/EGFr complex is due to the differential tyrosine phosphorylation of each receptor, as shown in Fig. 1. The amount of tyrosine phosphorylated EGFr/p185 complex associated with Shc, Grb2, and p72Raf proteins was estimated to be approximately 5% of the total tyrosine phosphorylated EGFr/p185 receptors. The kinetics of inducible association between the EGFr/p185$^{c\text{-}neu}$ heterodimer and these proteins was rapid occurring within 1 min of EGF treatment, only Shc demonstrated constitutive binding in the absence of EGF. Although p72Raf could form a complex with tyrosine-phosphorylated EGFr and p185 after EGF treatment, altered p72Raf mobility in NaDodSO$_4$-PAGE or increased p72 tyrosine phosphorylation (previously associated with the activation of p72Raf; (Morrison *et al.*, 1990) could not be demonstrated.

Grb2 does not appear to be a favored substrate for the EGFr tyrosine kinase (data not shown and Lowenstein *et al.*, 1992), and instead functions to link activated receptor tyrosine kinases to the mSOS1 protein (Egan *et al.*, 1993). However, the phosphorylation of Shc and formation of a protein complex composed of Sch and Grb2 have been shown to correlate with proliferative signals (Rozakis-Adcock *et al.*, 1993; Salcini *et al.*, 1994). To examine the role of SHC and GRB2 proteins in heterodimeric signaling proteins were immunoprecipitated after EGF treatment from a cell line expressing EGFr with wild-type p185 (M1) or cells expressing EGFr with the kinase inactive p185K757M (NEN757). Two proteins of molecular weight 46 and 52 kD contained low levels of phosphotyrosine from serum-starved cells, and showed increased phosphotyrosine content after EGF treatment in both cell lines (Fig. 5). These proteins were identified as p46 and p52 Shc by direct blotting with anti-SHC antisera. EGF-induced tyrosine phosphorylation of SHC was not negatively effected by the expression of the kinase-inactive p185K757M protein. The phosphorylation of p46 and p52 Shc correlated with the association with and tyrosine phosphorylation of the 170 to 180-kD EGFr/p185 complex (Fig. 5).

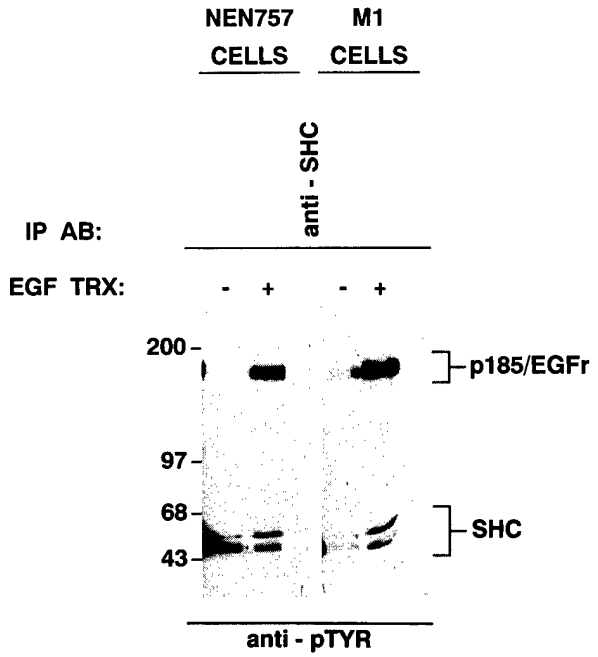

FIG. 5. Phosphorylation of SHC proteins. M1 cells that express both wild-type rat p185$^{c\text{-}neu}$ and human EGFr proteins or NEN757 cells, which express wild-type EGFr and a mutant form of p185$^{c\text{-}neu}$ (p185K757M) which lacking functional kinase activity, were treated with 100 ng/ml of EGF for 15 min and lysates were immunoprecipitated with polyclonal antisera against SHC as described in Materials and Methods. After NaDodSO$_4$-PAGE (8%) and transfer to nitrocellulose, the membrane was immunoblotted with anti-phosphotyrosine. Phosphorylated SHC proteins were visualized at 46, 52, and 66 kD.

Association of the heterodimeric EGFr/p185$^{c\text{-}neu}$ complex with PLC-g, PI-3 kinase, GAP, NCK, and SHPTP2

The formation of complexes between the EGFr/p185 heteromer and additional potential substrates including PLC-γ, the p85 subunit of phosphotidylinositol 3-kinase (PI3-kinase), NCK, the tyrosine phosphatase SHPTP2, and the GTPase activating protein (p120GAP) was examined next. The association and tyrosine phosphorylation of these proteins with activated receptors has previously been shown to correlate with positive, mitogenic signals (for review, see van der Geer *et al.*, 1994). The EGFr/p185$^{c\text{-}neu}$ heterodimeric complex (170–190 kD) associates with each of these substrate/effector proteins in an EGF-dependent fashion as illustrated by phosphotyrosine immunoblotting after anti-substrate immunoprecipitation (Fig. 6). Again, the presence of both EGFr and p185$^{c\text{-}neu}$ was confirmed by blotting with specific anti-receptor antisera. The association between receptor and substrate/effectors was ligand-inducible and rapid, occurring within 1 min.

The tyrosine phosphorylation of SH2-containing substrates was determined by analysis of the phosphotyrosine western blots. PLC-γ (145 kD) was phosphorylated by the heterodimer composed of EGFr and kinase inactive p185K757M, as demonstrated by the phosphorylation of a 145-kD band (Fig. 6, lane 6), which was subsequently demonstrated to be PLC-γ by blotting with PLC-γ-specific antisera (data not shown). The tyrosine phosphorylation of GAP (120 kD) was undetectable in anti-substrate immunoprecipitations. However, EGF-inducible tyrosine phosphorylation was demonstrated in these cells by Western blotting with substrate-specific antisera after anti-phosphotyrosine immunoprecipitation. p120GAP (Fig. 7A) showed enhanced tyrosine phosphorylation after EGF treatment. Phosphorylation of the p85 subunit of PI3-kinase was detectable at comparable levels in cell lines expressing EGFr with either wild-type p185 or kinase-inactive p185 (p185K757M) (Fig. 7B). The phosphorylation of the p72 SHPTP2 phosphatase could not be reproducibly demonstrated, even though phosphate inhibitors were included in the lysis and immunoprecipitations.

The p185$^{c\text{-}neu}$/EGFr heterodimer directly associates with the SH2 domains of PI3 kinase p85 subunit

Immunoprecipitation using antisera against PI-3 kinase also coprecipitated a phosphorylated protein of 150 kD in addition to the EGFr/p185c-*neu* heterodimeric complex present at 170–190 kD. Using Western blotting, this protein did not cross-

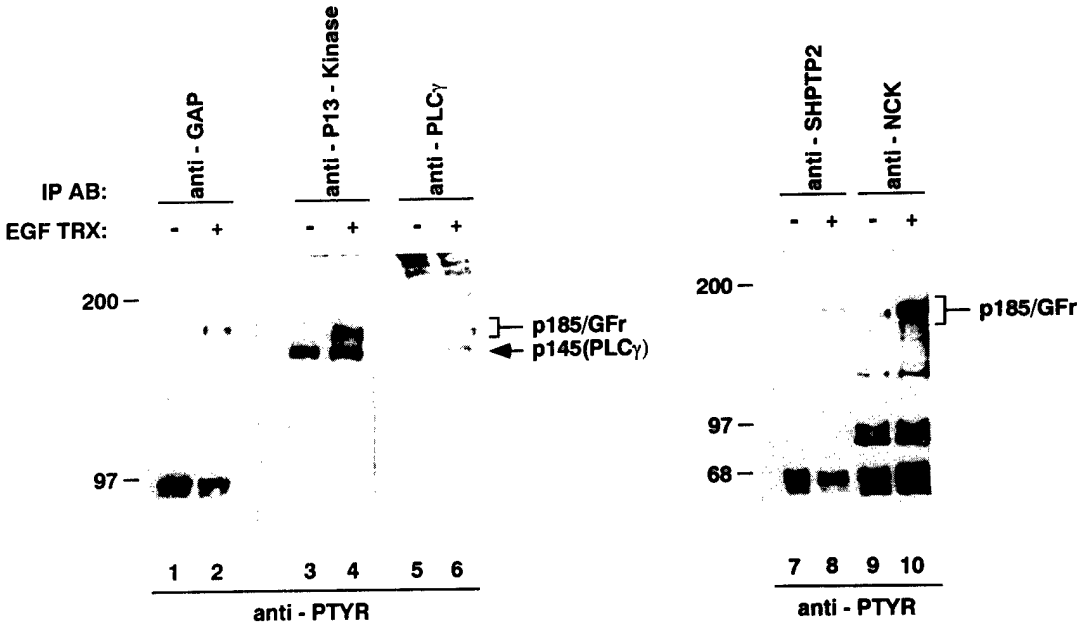

FIG. 6. EGF-induced association of the EGFr/p185 heterodimer with GAP, PI3-kinase, PLC-γ, SHPTP2, and NCK in NEN757 cells. Coprecipitation experiments were used to define association of cytoplasmic proteins with the EGFr/p185$^{c\text{-}neu}$ heterodimer. NEN757 cells were serum-starved overnight and treated with 100 ng/ml of EGF for 15 min as indicated. Triton X-100 (1%) lysates were immunoprecipitated with polyclonal antisera against the human GAP (lanes 1 and 2), rat PI3-kinase (lanes 3 and 4), bovine PLC-γ1 (lanes 5 and 6), and murine SHPTP2/SYP (tyrosine phosphatase) (lanes 7 and 8) and or monoclonal antibodies against the bovine NCK protein (lanes 9 and 10). Immunoprecipitations were processed as described in the Materials and Methods section and fractionated on $NaDodSO_4$-PAGE (8%) followed by immunoblotting with anti-phosphotyrosine antibodies. The phosphorylated EGFr and p185K757M proteins were visualized at 170–190 kD; the phosphorylated PLC-γ1 was visualized at 145 kD (lane 6). The constitutively phosphorylated 150-kD band in anti-PI3-kinase immunoprecipitates (lanes 3 and 4) was not identified.

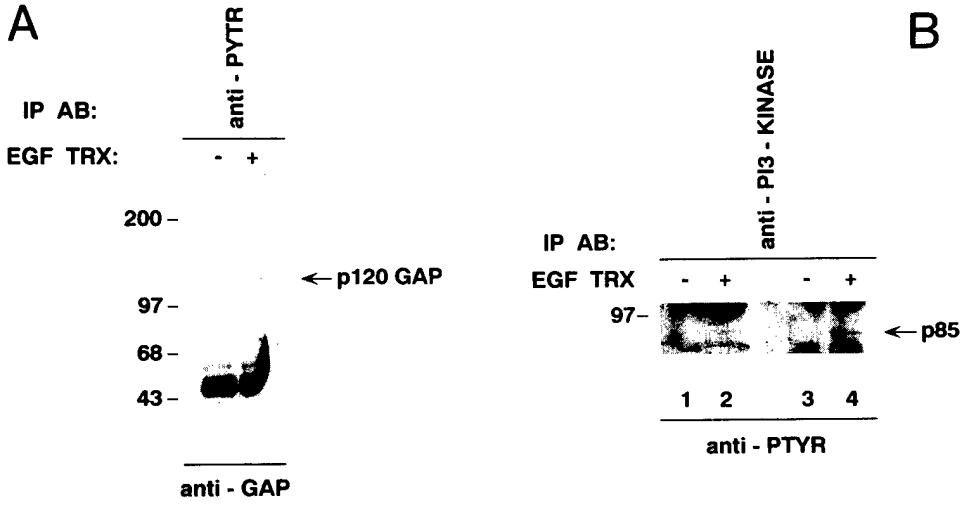

FIG. 7. EGF-induced phosphorylation of p120 GAP and the p85 subunit of PI3-kinase after EGF treatment of NEN757 or M1 cells. A. NEN757 cells were treated with 100 ng/ml of EGF for 15 min or left untreated as indicated, and total cellular lysates were immunoprecipitated with anti-phosphotyrosine antibodies. Immunoprecipitated complexes were fractionated on a 8% $NaDodSO_4$-PAGE and immunoblotted with polyclonal sera raised against GTPase-activating protein (GAP). The GAP protein was visualized at 120 kD. The prominent band at 55 kD is the Ig heavy chain. B. Either NEN757 cells (lanes 1 and 2) expressing wt EGFr with kinase-inactive p185K757M or M1 cells (lanes 3 and 4) expressing both wt EGFr and p185$^{c\text{-}neu}$ were serum-starved overnight, treated with 100 ng/ml of EGF for 15 min, and cellular lysates were prepared. Immunoprecipitation with anti-PI3-kinase antibodies was performed as described in Materials and Methods and was identical to that described for Fig. 5 (lanes 3 and 4). Proteins were fractionated by $NaDodSO_4$-PAGE and immunoblotted with anti-phosphotyrosine antibodies. Phosphotyrosine-containing proteins were detected by incubation with an anti-mouse IgG coupled to horseradish peroxidase and visualized by ECL (see Materials and Methods). The image was intentionally overexposed to detect faint signals. The phosphorylated p85 subunit of PI3-kinase was visualized at 85 kD after EGF treatment in both cell lines.

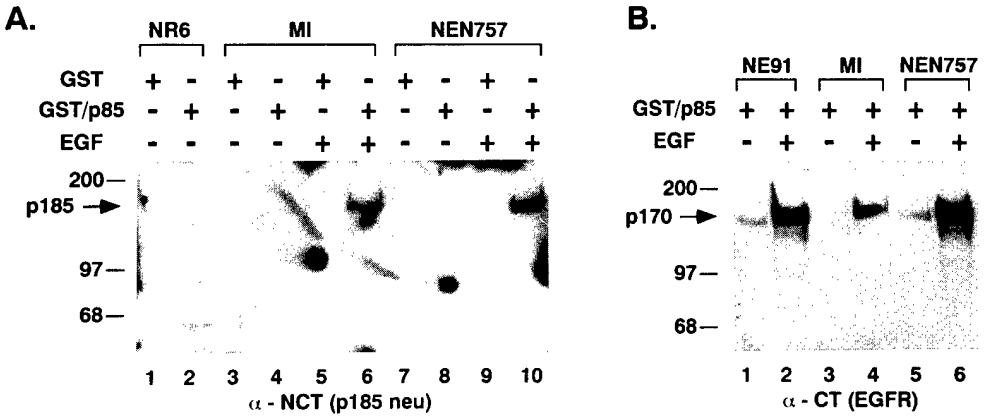

FIG. 8. Association of p85PI3 kinase SH2 domains with both the EGFr and p185 forms after EGF treatment A. Association of the p85 SH2 domains with the tyrosine phosphorylated EGFr and p185K757M proteins was determined using GST-fusion protein binding and anti-p185 immunoblotting with anti-NCT antisera as described in the Methods and Materials. Lanes 1 and 2, Reactivity of GST/p85 SH2 domains with proteins in the parental NR6 cell line, which lacks endogenous EGFr and $p185^{c\text{-}neu}$; lanes 3 and 4, binding of GST/p85 SH2 proteins with p185 protein in the M1 cell lines, which express both wild-type EGFr and p185; lanes 5 and 6, binding to p185 in the NEN757 cell line, which expresses EGFr with the kinase-inactive p185K757M. B. Association of GST/p85 SH2 domains with the EGFr was determined using antiserum (CT) specific for EGFr in the Western blotting. Lanes 1 and 2, EGF-inducible binding in a control cell line NE91, which expresses only EGFr; lanes 3 and 4, bining of the GST/p85 SH2 protein in the M1 cells; binding to EGFr in NEN757 cells. Each cell line represented expresses approximately equivalent EGFr or p185 protein ($2.5–7.5 \times 10^5$ receptors/cell) (Qian *et al.*, 1994a).

react with PLC-γ antisera or antisera raised against the p85 subunit of PI3 kinase (data not shown). To demonstrate the specificity of interaction between PI3 kinase and the EGFr/p185 heterodimer, the ability of the receptor proteins to bind glutathione-*S*-transferase (GST) fusion proteins containing the amino- and carboxy-terminal SH2 domains of the p85 subunit of PI3 kinase (GST/p85) was examined.

GST/p85 fusion protein or control GST proteins without p85 SH2 domains were purified from bacteria and added to cell lysates following EGF treatment. Association of the GST/p85 SH2 protein with EGFr or $p185^{c\text{-}neu}$ was measured by Western blotting using receptor-specific antisera (Fig. 8). EGF treatment for 15 minutes mediated an increased association of GST/p85 SH2 domains with both wild-type $p185^{c\text{-}neu}$ expressed in M1 cells (Fig. 8A, lanes 4 and 6) and the kinase-inactive p185K757M expressed in NEN757 cells (lanes 8 and 10). Comparable levels of either wild-type p185 or p185K757M were coprecipitated with GST/p85 after activation and phosphorylation after EGF treatment (compare lanes 6 and 10).

In a similar fashion, EGF treatment induced the efficient association between EGFr and p85 SH2 protein in the same cell lines (Fig. 8B, lanes 3–6). There was no apparent reduction in the amount of EGFr associated with GST/p85 from NEN757 cells (EGFr expressed with p185K757M), M1 cells (wild-type EGFr and p185), or NE91 cells (wild-type EGFr only). The relative total amounts of EGFr expressed in the cell lines were approximately equal (Qian *et al.*, 1994a and data not shown). The control GST protein did not specifically bind either EGFr or $p185^{c\text{-}neu}$ forms.

DISCUSSION

Ligand-induced dimerization or oligomerization is a requisite step in the activation and signaling of most growth factor receptors (Weiner *et al.*, 1989 and reviewed in Heldin, 1995). Heterodimerization (or oligomerization) of related tyrosine kinase receptors may profoundly expand the potential repertoire of substrate binding sites and subsequent substrate activation. The intermolecular association of the EGFr cytoplasmic domain with the p185 tyrosine kinase has been shown to enhance substrate binding and transformation (Qian *et al.*, 1995) suggesting the amplification of tyrosine kinase signaling through additional substrate binding site availability. Likewise, p160c-erbB-3, which has a low or nonexistent kinase activity (Prigent and Gullick, 1994), provides additional binding motifs for the p85 subunit of PI3-kinase that may be amplified through heterodimerization of p160c-erbB-3 and p185c-erbB-2 (Carraway and Cantley, 1994; Soltoff *et al.*, 1994). The heterodimerization between p160c-erbB-3 and p185c-erbB-2 or p160c-erbB-3 and p180c-erbB-4 also may provide for high-affinity binding of the heregulin family of ligands (Plowman *et al.*, 1993; Slikowski *et al.*, 1994). Heterodimerization within the EGFr family thus appears to be a general mechanism that provides for an increased intensity or diversity of ligand-induced signaling responses (Kokai *et al.*, 1989; Wada *et al.*, 1990; and reviewed in Lemmon and Schlessinger, 1994).

Heterodimerization between the EGFr and $p185^{c\text{-}neu}$ tyrosine kinase receptors has been well characterized in transfected mouse cell lines (Wada *et al.*, 1990; Spivak-Kroizman *et al.*, 1992) and human adenocarcinoma cell lines (Goldman *et al.*, 1990). The significance of the formation of this heterodimeric complex between EGFr family members is indicated by: (i) the resulting transformation of fibroblasts in the absence of exogenously added growth factor (Kokai *et al.*, 1989); (ii) the predominance of heterodimeric EGFr/p185 species over homodimeric forms (Qian *et al.*, 1994a); and (ii) the loss of EGF-induced responses in cell lines coexpressing wt EGFr with a kinase-inactive form of p185 (Qian *et al.*, 1994a,b, and this paper). To explore the potential defects in the signal transduc-

tion pathway(s) mediated by EGFr and a kinase-inactive p185 heterodimer, we characterized the receptor association and phosphorylation of several known substrates for receptor tyrosine kinases.

The hypothesis that EGFr and p185$^{c\text{-}neu}$ can couple with distinct signal transduction pathways has been based on several observations. The distinct phosphorylation patterns of intracellular substrates by EGFr and p185 tyrosine kinases (Fazioli *et al.*, 1991) argues for the direct phosphorylation of unique substrates including the EGFr-specific eps15 (Fazioli *et al.*, 1993), or the activation of unique pathways by the two receptors. Moreover, the two receptors have demonstrated differential mitogenic potency in different cell lines (Di Fiore *et al.*, 1990), suggesting that the utilization of alternative substrate pathways is important for signaling through members of the EGFr family.

In the model system used in the present study, we derived cell lines transfected with EGFr or p185 forms from a common parental clone, therefore there should be no differences in the content of intracellular kinase substrates. The coexpression of EGFr with either wild-type p185 or the kinase inactive p185K757M had profound effects on the mitogenic or transforming potency of EGF signaling. Expression of the p185K757M protein with EGFr has been shown to functionally inactivate EGF-inducible DNA synthesis, receptor turnover, and cellular transformation in NEN757 cells (Qian *et al.*, 1994b). However, the present study demonstrates that the EGFr tyrosine kinase in these cells was still active and capable not only of phosphorylating p185 but was also active in total cellular substrate phosphorylation (Figs. 1 and 2). The dose-dependent response to EGF in terms of tyrosine phosphorylation was qualitatively and quantitatively the same whether wild-type p185 (M1 cells) or the kinase-inactive form (NEN757 cells) was expressed with EGFr (Fig. 2). The defective mitogenic signaling in NEN757 cells was then not due to a disruption of tyrosine kinase and phosphorylation activity, but rather may be due to the loss of phosphorylation or activation of a specific subset of intracellular signal transducers. The possibility that the activity of the p185 tyrosine kinase coexpressed with EGFr couples with alternative substrate pathways is suggested by the phosphorylation of different substrate proteins, as shown in Fig. 3.

Considerable effort has demonstrated that the phosphorylation and activation of putative tyrosine kinase substrates correlates with positive mitogenic signals. However, it is unclear whether the protein–protein association between substrate and activated receptor or the actual substrate phosphorylation is a more important determinant for substrate activation. PLC-γ1 and PI3 kinase have been shown to be associated with activated receptors and subsequently phosphorylated and activated by receptor tyrosine kinases (Nishibe *et al.*, 1990). p120 GAP has also been shown to be associated with activated tyrosine kinase receptors (Ellis *et al.*, 1990) and to become phosphorylated (Morrison *et al.*, 1990). In the present study, PLC-γ1, the p85 subunit of PI3-kinase, Shc, and p120 GAP became associated with the phosphorylated EGFr/p185K757M complex and were phosphorylated themselves after EGF treatment (Figs. 4 and 6). The stoichiometry of substrate phosphorylation appeared to be equally efficient in cells with the kinase inactive p185K757M as with the wild-type p185 (Figs. 5 and 7). However, the enzymatic activation of the respective substrate proteins was not analyzed directly. Shc, NCK, and Grb2 were shown to form EGF-inducible complexes with the EGFr/p185 heterodimer, suggesting that these adapter proteins can functionally couple with downstream signals (Fig. 4 and 6).

Because the catalytic activity of SHPTP2 has been demonstrated to be enhanced by an EGFr-dependent phosphorylation (Lechleider *et al.*, 1993; Vogel *et al.*, 1993) and an inhibitory antibody against SHPTP2 reduced EGF-stimulated DNA synthesis (Xiao *et al.*, 1994), this substrate protein is a good candidate for further analysis in the defective signaling pathway presented here. The tyrosine phosphatase SHPTP2 also became associated with the phosphorylated EGFr/p185K757M complex (Fig. 6); however, increased phosphotyrosine content on SHPTP2 could not be detected. The specific activation of p72RAF has also been correlated with growth factor activation (Morrison *et al.*, 1990). Further analysis of the activation status of p72RAF and SHPTP2 in NEN757 cells is currently being performed.

An additional explanation for the defect responsible for the inactivated EGF response in these cells is the improper subcellular localization or enzymatic activation of each of these putative substrate proteins. The association of PLC-γ1, p120GAP, PI3-kinase, and p72RAF with the membrane-bound receptor proteins suggests that these substrates are properly located for their respective activities. It has been demonstrated that the subcellular localization of a complex between tyrosine-phosphorylated Shc, Grb2, and mSOS correlates with distinct signaling responses (Di Gugliemo *et al.*, 1994); the proper association of p72Raf, Grb2, and Shc with the membrane-bound receptor complex (Fig. 4) also suggests that substrate localization is not an issue with the NEN757 cells.

The particular relevance of individual substrates for certain signaling pathways is questionable. For instance, the relative mitogenic potency of EGFr and p185 does not necessarily correlate with the relative phosphorylation levels of two substrates p120GAP and PLC-γ1 (Fazioli *et al.*, 1991). There have been several observations of alternative EGF-dependent signaling pathways that may require uncharacterized substrate/enzyme pathways. Some initial findings of EGFr tyrosine kinase-independent pathways (Campos-Gonzales and Glenney, 1992; Selva *et al.*, 1993) may be due to activation of endogenous murine EGFr in transfected cells (Hack *et al.*, 1993). However it is impossible to exclude as yet unidentified EGF-activated kinases. The particular relevance of the JAK/STAT pathway for EGFr-mediated signaling is still not clear, although in preliminary analysis we have demonstrated EGF-inducible association of stat1α and stat3 with the EGFr/p185K757M complex as well as EGF-inducible activation of STAT DNA binding activity (Dougall, Samanta, and Greene, manuscript in preparation), indicating that the JAK/STATs are probably not essential for EGFr signaling.

DiFiore and colleagues have identified novel substrates by virtue of their tyrosine phosphorylation after EGF treatment (Fazioli *et al.*, 1992). Two of these substrates (eps8 and eps15) have been shown (in the NEN757 cells used in the present study) to become tyrosine phosphorylated and coprecipitate with EGFr and p185K757M in an EGF-dependent manner (W. Dougall, W. Wong, and M. Greene, unpublished observations).

The transforming capacity of the p185 tyrosine kinase has

been linked to the activation of the MAP kinase and *ras* pathway (Ben-Levy *et al.*, 1994). The specific activation of the ERK family of MAP kinases is likely to be a critical trigger in the signaling through receptor tyrosine kinases (Marshall, 1995). The distinction between mitogenic/transformation signals and differentiation signals may be mediated by the duration of ERK activation. Direct analyses of the activation of the MAP kinase enzymes were not performed in the present study, however, we demonstrated that three proteins involved in the upstream regulation of the MAP kinase pathway (p72Raf, Grb2, and Shc) are correctly associated with the heterodimer expressed in the NEN757 cells.

Future studies in this model system will focus on a comparison between EGF-responsive and nonresponsive EGFr/p185 heterodimers with regard to specific activation of MAP kinases. It is interesting to note that the EGF-inducible internalization rate of the complex formed between EGFr and p185K757M is significantly retarded (as compared to a complex between wild-type proteins) (Qian *et al.*, 1994b), raising the possibility that the activation of downstream signaling pathways is improperly sustained at the plasma membrane, thereby altering the normal mitogenic signal. Alternatively, certain signals initiated by the p185/EGFr heterodimer may be routed through heterologous kinase pathways independent of the MAPK and *ras* pathways, as has been observed for EGF-dependent signaling in neuroendocrine cells (Pickett and Gutierrez-Hartmann, 1994).

Several questions remain as to the nature of the functional inactivation of EGF-inducible DNA synthesis, receptor turnover, and transformation seen in NEN757 cells coexpressing EGFr with the kinase-inactive p185K757M. The present study demonstrates that the activation of the EGFr tyrosine kinase and the tyrosine phosphorylation of several substrates proceeds in an accurate and stoichiometric manner in cells with abnormal heterodimeric receptors. Thus, substrate association and phosphorylation do not stringently correlate with the mitogenic and transforming activity of this receptor complex, suggesting additional pathways or mechanisms vital to EGFr/p185$^{c\text{-}neu}$ heterodimeric signaling. It is also likely that the defective signaling may then be attributable to defects in the specific activation of downstream enzymes or effector molecules.

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Address reprint requests to:
Dr. Mark I. Greene
Center for Receptor Biology and Division of Immunology
Department of Pathology and Laboratory Medicine
University of Pennsylvania School of Medicine
Philadelphia, PA 19104

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Structural analysis of p185$^{c\text{-}neu}$ and epidermal growth factor receptor tyrosine kinases: Oligomerization of kinase domains

(receptor dimerization/molecular modeling/enzyme structure-function/autophosphorylation)

RAMACHANDRAN MURALI*, PATRICK J. BRENNAN, THOMAS KIEBER-EMMONS, AND MARK I. GREENE

Department of Pathology and Laboratory of Medicine, University of Pennsylvania, Philadelphia, PA 19104

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ABSTRACT **The epidermal growth factor receptor (EGFR) and p185$^{c\text{-}neu}$ proteins associate as dimers to create an efficient signaling assembly. Overexpression of these receptors together enhances their intrinsic kinase activity and concomitantly results in oncogenic cellular transformation. The ectodomain is able to stabilize the dimer, whereas the kinase domain mediates biological activity. Here we analyze potential interactions of the cytoplasmic kinase domains of the EGFR and p185$^{c\text{-}neu}$ tyrosine kinases by homology molecular modeling. This analysis indicates that kinase domains can associate as dimers and, based on intermolecular interaction calculations, that heterodimer formation is favored over homodimers. The study also predicts that the self-autophosphorylation sites located within the kinase domains are not likely to interfere with tyrosine kinase activity, but may regulate the selection of substrates, thereby modulating signal transduction. In addition, the models suggest that the kinase domains of EGFR and p185$^{c\text{-}neu}$ can undergo higher order aggregation such as the formation of tetramers. Formation of tetrameric complexes may explain some of the experimentally observed features of their ligand affinity and hetero-receptor internalization.**

p185$^{c\text{-}neu}$ and its human homologue p185$^{c\text{-}erbB\text{-}2}$ are related to the epidermal growth factor receptor (EGFR) and possess intrinsic tyrosine kinase activity. Overexpression of these receptors has been correlated with poor prognosis of human adenocarcinoma of breast, ovarian, and pancreatic cancers (1–3). These receptor-tyrosine kinases (RTK) are characterized by an ectodomain, a transmembrane domain, and an endodomain. The endodomain consists of a kinase domain and a carboxyl-terminal tail, which contains most of the autophosphorylation sites (4). Many RTK receptors, except insulin receptor, appear to undergo oligomerization upon ligand binding (4–6). Oligomerization can occur between the same receptors, forming a homodimer, or different members of the same receptor family, thus forming a heterodimer (6, 13). When ligand binds to the ectodomain of the receptor, conformational changes are propagated through the transmembrane domain to the cytoplasmic domain, resulting in receptor activation (7). The net effects of oligomerization are enhanced kinase activity and initiation of signal transduction (5). A similar scenario has been proposed for several cytokines and their receptor complexes (6). Thus, oligomerization seems to be a common pathway leading to signal propagation (7).

The ectodomain has been postulated to bring the cytoplasmic domains into proximity and orient them for favorable kinase activity (4, 8). The role of the transmembrane region in RTK seems to stabilize the cytoplasmic domain (4). p185$^{c\text{-}neu}$, on the other hand, has been shown to undergo dimerization caused by a point mutation (Val-664 → Glu) in the transmembrane region (8, 9). An analogous transmembrane position in p185$^{c\text{-}erbB\text{-}2}$, mutation of Val-659 → Glu, also leads to dimerization (10). The linker region between the transmembrane region and the kinase domain seems relevant for kinase activity, though no specific role has yet been assigned to this area. Mutations in this region appear to affect kinase activity (4).

p185$^{c\text{-}neu}$ and EGFR can associate and undergo dimerization (11–13). Upon dimerization, enhanced kinase activity ensues and the receptors transphosphorylate each other (12, 14). The ectodomain is necessary for association of p185$^{c\text{-}neu}$ and EGFR, and this heteromer formation is increased in a ligand-dependent manner (12, 14). An active heterodimeric complex of EGFR and p185$^{c\text{-}neu}$ proteins was able to contribute to cellular transformation (12–15). However, cells transfected with EGFR and a kinase-deficient p185$^{c\text{-}neu\ K758M}$ inhibited the kinase activity of EGFR, abolished cellular transformation, and reduced EGF-stimulated mitogenesis (14, 16). These results further suggest that kinase activation requires an association of the cytoplasmic domains of the respective receptors, and that this feature is indispensable to receptor activation and signal transduction.

Although the ectodomain has been shown to be involved in oligomerization, it is not known whether the kinase domains can form dimers in the cytoplasm of cell. We have explored this aspect by using homology modeling. We have analyzed structural features of the monomeric, dimeric, and tetrameric forms of p185$^{c\text{-}neu}$ and EGFR kinase domains. Our study shows that formation of dimers and tetramers within cells is plausible, and this oligomeric state might be a strategy used to recruit diverse substrates in a structure dependent manner and may, as suggested (13), represent a novel diversification mechanism for nonpolymorphic receptors.

MATERIALS AND METHODS

The sequences of the kinase domain of p185$^{c\text{-}neu}$, EGFR, and cAMP-dependent kinase (cAPK) were aligned using the multiple sequence alignment program CLUSTALV (17). The alignment was then adjusted manually, conserving the overall secondary structure and positioning the residues known to be critical in binding adenosine triphosphate (ATP) and in the catalytic site. We modeled both p185$^{c\text{-}neu}$ and EGFR by using the crystal structure of cAPK (18). The modeling was carried out by using QUANTA (Molecular Simulations, Cambridge, MA), and energy minimization was performed using both QUANTA and XPLOR 3.1 (19). CHARMM energy parameters were used in both the programs. The models were analyzed by using QUANTA, O (20), and GRASP (21).

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Abbreviations: RTK, receptor tyrosine kinase(s); EGF, epidermal growth factor; EGFR, EGF receptor; cAPK, cAMP-dependent kinase; rms, root mean square; ATP, adenosine triphosphate; IRK, insulin receptor kinase.

*To whom reprint requests should be addressed.

A trial structure was built based on the sequence alignment. Insertions, deletions, and mutations were incorporated into the template structure to build an initial model. For both p185$^{c\text{-}neu}$ and EGFR, the sequence alignment led to trial structures with two large insertions and four small deletions in the kinase domain. At the loops where insertions and deletions occur, we used a knowledge-based approach for loop conformation construction (22, 23) because the sizes of the loops were small (five to six residues). The structural data base was created with QUANTA by using the latest coordinates from the Brookhaven National Laboratory data bank. The search was performed with a minimum of two residues included at the termini. The fragments were selected based upon sequence homology and the avoidance of steric contacts. When several satisfactory loops were found, an average structure was accepted. The fragments were annealed and regularized to the template by using QUANTA. The trial structure, with insertion fragments and deletions, was then subjected to energy minimization followed by molecular dynamics. Mutated side chains were screened for steric contacts and, if necessary, remodeled either by using the rotamer library data base (24) or by manually rotating the side chains.

The regions of insertion and deletion were minimized while holding the remainder of the structure fixed, thus preserving the overall structure. The entire structure was then subjected to conjugate gradient energy minimization for 2000 cycles to convergence, followed by an equilibration and production run of molecular dynamics at 300 K for 60 ps. Molecular dynamics was performed to remove any steric contacts and to allow a change in the conformation of inserted loops, if more favorable. All energy calculations were performed at a dielectric constant of 1. Final energy values were calculated by using CHARMM for monomers and XPLOR 3.1 (19) for oligomers. Average root mean square (rms) deviations between the alpha carbon positions of the final models were calculated by using QUANTA. Parameters for MnATP were built from CHARMM and used in all calculations. The consistency of the model was checked by using profile 3-D (25).

Aggregation states were predicted through manual docking of one subunit while holding the remaining subunit(s) fixed. The best-shape complementarity between the kinase domains was obtained by rotation of 165° and translation of 10–12 Å of one of the monomers with respect to its counterpart. The area of surface buried was calculated by using QUANTA with a probe radius of 1.4 Å. Number and type of contacts were evaluated by the program CONTACT (26), with a distance cut-off of 4.5 Å. A tetramer was constructed from the homodimers with 2-fold symmetry operation about an axis parallel to the pseudo-dimer axis of the homodimers. Individual monomers were then adjusted to maximize the complementarity, followed by 40 cycles of rigid body minimization by using XPLOR (19). Electrostatic calculations were performed with GRASP. Charged amino acid groups were assigned full charges as provided in GRASP and charges for MnATP were obtained from CHARMM19, which is part of QUANTA. The electrostatic calculations were performed with distance-dependent dielectric constants, from 1 at the interior to 80 at the outer surface.

RESULTS

Structural and Sequence Alignment of Tyrosine Kinase Domains. Crystal structures of cAPK with and without MgATP show that the ATP binding domain can rotate away from the catalytic domain such that the cleft is either wide open or closed (27). Thus, the kinase domain may exist in either an open form (inactive) or closed form (active). While the active form of cAPK requires bound MgATP, we have shown that Mn^{2+} is preferred over Mg^{2+} by p185$^{c\text{-}neu}$ and EGFR for kinase activity (28). We therefore used MnATP for our calculations. The residues in the ATP binding domain and catalytic region are conserved in all kinases but the activation loop (184–200 of cAPK) is the most variable region among kinases. Choosing an alignment for the autophosphorylation tyrosine residues in p185$^{c\text{-}neu}$ and EGFR (Tyr-882 in p185$^{c\text{-}neu}$ and Tyr-845 in EGFR) proved difficult, and the tyrosines were aligned in two ways within the activation loop: the tyrosine residues were aligned with Thr-197 of cAPK, shown to be phosphorylated in the crystal structure, and alternatively, as an insert in the activation loop (Fig. 1). In both cases, the conformation of the activation loop and the orientation of tyrosines were very similar, suggesting that the alignment of these tyrosines was not critical. The final alignment (Fig. 1) is in agreement with an alignment developed by Knighton *et al.* (29).

Tyrosine Kinase Domain. The overall structure of the kinase domain in p185$^{c\text{-}neu}$ and EGFR is very similar to that of cAPK. The conserved parts of EGFR and p185$^{c\text{-}neu}$ have an rms deviation of 0.43 Å and 0.34 Å and the overall rms deviation is 1.9 Å (Fig. 2). The secondary structures are

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cAPK   43  FDRIKILGTGSFGRVMLVKHKESG----NHYAMKI  73
EGFR  688  FKKIKVLGSGAFGTVYKGLWIPEGEKVKIPVAIKE 722
Neu   725  LRKVKVLGSGAFGTVYKGIWIPDGENVKIPVAIKV 759

cAPK   74  LDKQKVVKLKQIEHTLNEKRILQAVNFPFLVKLEF 108
EGFR  723  LREATSP--KANKEILDEAYVMASVDNPHVCRLLG 755
Neu   760  LRENTSP--KANKEILDEAYVMAGVGSPYVSRLLG 792

cAPK  109  SFKDNSNLYMVMEYVAGGEMFSHLRRIGRFSEPHA 143
EGFR  756  ICLTSTVQLITQLMPFGCLLDYVREHKDNIGSQYL 790
Neu   793  ICLTSTVQLVTQLMPYGCLLDHVRENRGRLGSQDL 827

cAPK  144  RFYAAQIVLTFEYLHSLDLIYRDLKPENLLIDQQG 178
EGFR  791  LNWCVQIAKGMNYLEDRRLVHRDLAARNVLVKTPQ 825
Neu   828  LNWCVQIAKGMSYLEDVRLVHRDLAARNVLVKSPN 862

cAPK  179  YIQVTDFGFAKRVKGRTWT-----LCGTPEYLAPE 208
EGFR  826  HVKITDFGLAKLLGAEEKEYHAEGGKVPIKWMALE 860
Neu   863  HVKITDFGLARLLDIDETEYHADGGKVPIKWMALE 897

cAPK  209  IILSKGYNKAVDWWALGVLIYEMAAGYPPFFADQP 243
EGFR  861  SILHRIYTHQSDVWSYGVTVWELMTFGSKPYDGIP 895
Neu   898  SILRRRFTHQSDVWSYGVTVWELMTFGAKPYDGIP 932

cAPK  244  IQIYEKIVSGKVRFPSH-FSSDLKDLLRNLLQV-D 276
EGFR  896  ASEISSILEKGERLPQPPICTIDVYMIMVKCWMID 930
Neu   933  AREIPDLLEKGERLPQPPICTIDVYMIMVKCWMID 967

cAPK  277  LTKRFGNLKNGVNDIKNHKWF               297
EGFR  931  ADSRP-KFRELIIEFSKMARD               950
Neu   968  SECRP-RFRELVSEFSRMARD               987
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FIG. 1. Sequence alignment of cAPK, p185$^{c\text{-}neu}$, and EGFR. The amino acid sequences were aligned based on the crystal structure of cAPK (18). The alignment was made such that the secondary structures were conserved. The insertions and deletions were allowed only at the loops. The identical amino acids are boxed.

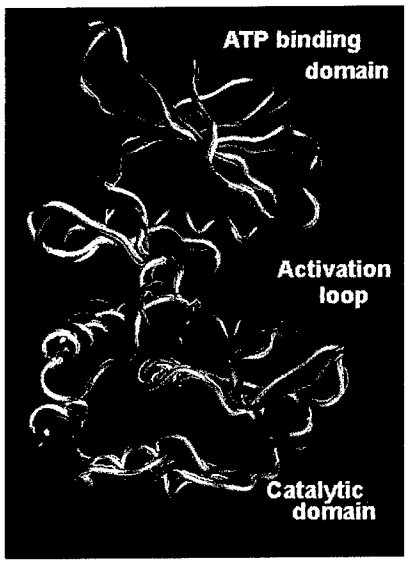

FIG. 2. Comparison of the models of p185$^{c\text{-}neu}$ and EGFR kinase domains. p185$^{c\text{-}neu}$ (red) and EGFR (magenta) kinase domain are shown as trace. The kinase domain is comprised of a smaller lobe or a ATP binding domain and a catalytic domain. The activation loop is located between catalytic domain and the ATP binding domain. Overall secondary structure is similar in both the models of p185$^{c\text{-}neu}$ and EGFR kinase domains. The most variable and flexible region is the activation loop where the autophosphorylation tyrosines are located. The ATP binding domain (the small lobe) is more conserved than the catalytic domain.

conserved in EGFR, p185$^{c\text{-}neu}$, and cAPK with only minor changes. Naming of secondary structures follows that used for cAPK (18). The smaller lobe at the N terminus or ATP binding domain is rich in β strands, and the large lobe at the C terminus or the catalytic domain is rich in α helices. Based upon the alignment, the p185$^{c\text{-}neu}$ and EGFR models lack the β1 strand, β4 is short, and the β5 strand is longer. The region β4 to β5 contains sequences that are not conserved with respect to cAPK. One of the two helices observed in the small lobe, the αB helix, is distorted from that seen in cAPK in both p185$^{c\text{-}neu}$ and EGFR due to the insertions in that region. The helix αD, which is close to the hinge region in cAPK, is also distorted in p185$^{c\text{-}neu}$ and EGFR.

Homodimers and Heterodimers. *Substrate binding region.* Dimers are stabilized by β strands at the top and by the two helices αEF and αG at the bottom (Fig. 3*c*). The homodimers of p185$^{c\text{-}neu}$ and EGFR are shown in Fig. 3 *a* and *b*, and the heterodimers are shown in Fig. 3*d*, which shows that dimers have a similar interface geometry. In our model, the heterodimer interface is formed by six segments from p185$^{c\text{-}neu}$ (727–740, 760–771, 795–799, 880–891, 899–901, and 929–937) and five segments from EGFR (696–701, 722–731, 845–864, and 891–904). The substrate binding region in the dimers is formed at the interface of the two monomers with their MnATP facing each other. Both the heterodime and p185$^{c\text{-}neu}$ homodimer have similar binding pocket size. The substrate binding region is 20.6 Å wide, and the interior narrows down to 7.2 Å and then widens in the back to 10.2 Å. Distance between the dimers (as measured from C$^{\gamma}$ of ATP) is about 20.6 Å. The EGFR-homodimer is 28.9 Å wide near the substrate binding region and narrows down to 14.2 Å near the center of the cavity and in the back widens to 17.9 Å. The EGFR dimers are separated by 16.1 Å.

Association of dimers and tetramers. Studies on protein–protein interaction have identified three major characteristics associated with these interactions: complementarity of shapes, a large area buried (or loss of accessible surface area upon dimerization), and finally, contacts are often dominated by hydrophobic interactions (30, 31). In this study we assumed minimal structural change in the monomer proteins upon oligomerization. The oligomerization of p185$^{c\text{-}neu}$ and EGFR was analyzed by using conformational energy calculations and accessible surface area. The results obtained from the calculations of accessible surface area for the oligomers and molecular energy calculation are shown in Table 1. Surprisingly, the nature of the surface at the interface upon oligomerization is very similar in both homodimers and the heterodimer. The surface is predominately formed by nonpolar atoms at the interface. Heterodimer association leads to a loss of surface area, which is about 2% greater than in the homodimers. In addition, the total energy of the heterodimer is lower than that of the homodimers (Table 1). Clearly, the total energy is dominated by electrostatic interactions. This major electrostatic energy was contributed by the clustering of charges on the surface and the interior of the molecules. The core is negatively charged, and one end of the molecule contains a positively charged cluster (Fig. 4). In the heterodimer, the positive charges span from one side of EGFR to the interface of the p185$^{c\text{-}neu}$. The positively charged surface patch is formed by residues from EGFR (His-749, Arg-752, Lys-822, and His-826) and p185$^{c\text{-}neu}$ (Arg-726, Lys-727, and His-818). The features of p185$^{c\text{-}neu}$ and EGFR tetramers are similar to that of dimers (data not shown). Energetic and surface area calculations suggest that the tetrameric association of p185$^{c\text{-}neu}$ and EGFR is less stable than the heterodimer.

DISCUSSION

Protein kinases share a high degree of homology in the catalytic domain, whether they are Ser/Thr kinases or tyrosine kinases (32). Several crystal structures of Ser/Thr kinases have now been determined, including the cAMP dependent kinase (18), mitogen-activating kinase ERK-2 (33), twitchin kinase (34). Recently, the kinase domain of insulin receptor (IRK), a member of the tyrosine kinase family was determined (35). These protein structures reveal that the kinase domains not only share sequence homology but also have very similar topologies. Sequence homology among the kinase family members has helped to develop an informative model of the EGFR kinase domain (29). p185$^{c\text{-}neu}$ belongs to class I type receptors and is very similar to EGFR, sharing 82% amino acid sequence homology in the tyrosine kinase domain.

The ATP binding domain is mostly conserved in both cAMP-dependent kinases and in the kinase domains of RTK. The sequence alignments of kinases show that the Ser/Thr kinases contain a flexible hinge sequence (Gly-125–Gly-126) near the ATP binding region (Fig. 1). This has been replaced by Gly–Cys in tyrosine kinases. Structurally, this indicates that p185$^{c\text{-}neu}$ and EGFR have less freedom to open and close than Gly–Gly-containing kinases. In comparison to cAPK (a Ser/Thr kinase), there is an additional helix, disposed near the activation loop of p185$^{c\text{-}neu}$ and the EGFR tyrosine kinases. The minor changes in the secondary structural features of p185$^{c\text{-}neu}$ and EGFR kinase domains are consistent with characteristics observed in both the IRK (35) and EGFR models (29). The amino acid residues involved in ATP binding are conserved in all the tyrosine kinases, including p185$^{c\text{-}neu}$ and EGFR. In p185$^{c\text{-}neu}$, the ligand ATP is neutralized by two charged residues, Lys-758 and Arg-854, in comparison to only Lys-72 in cAPK. The role of Lys-758 in p185$^{c\text{-}neu}$ has been shown to be critical for kinase activity. Mutation of this residue abolishes the kinase activity and also abrogates cellular transformation (9). In EGFR, Lys-721 and Arg-817 may play a similar role.

The catalytic site, where phospho-group transfer occurs, is located between the ATP binding and catalytic domains. The putative catalytic site in p185$^{c\text{-}neu}$ is formed by Lys-758, Glu-775, Asp-850, Arg-854, and Asp-868, as discerned from

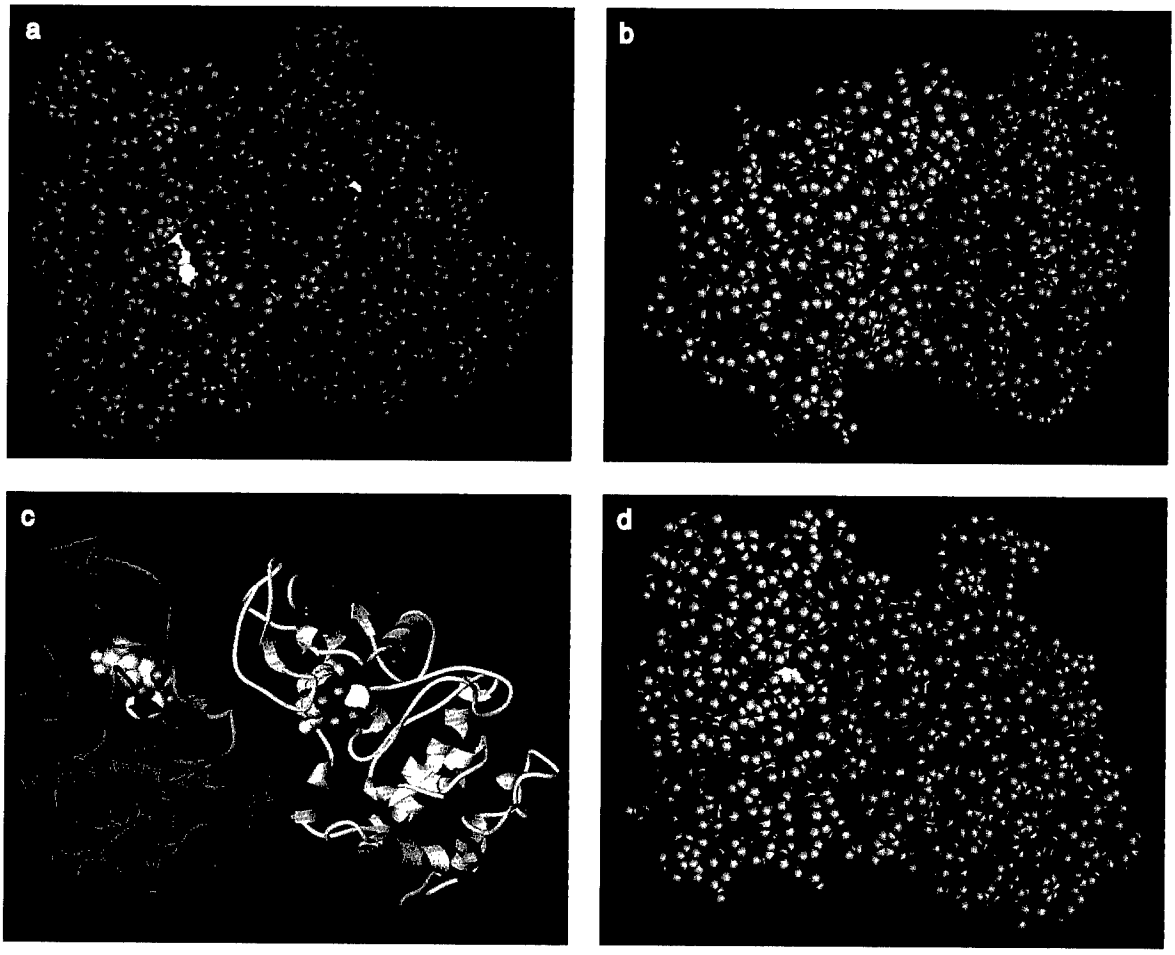

FIG. 3. Oligomerization of the kinase domains of p185$^{c\text{-}neu}$ and EGFR. The molecules are viewed down the autophosphorylation tyrosines. Each molecule within a given homodimer is shown in different color. The autophosphorylation tyrosine is shown in pink, and MnATP moiety is shown in yellow. The space-filling model of p185$^{c\text{-}neu}$ homodimer shows that the autophosphorylation tyrosine is close to the substrate binding domain and fully exposed (*a*), while in homodimer of EGFR the autophosphorylation site is partially buried and much closer to the substrate binding region (*b*) (see text for discussion). (*c*) Heterodimer formation is shown as ribbon model to highlight the complementarity between the p185$^{c\text{-}neu}$ and EGFR. The p185$^{c\text{-}neu}$ monomer is shown in purple and EGFR by yellow. The two molecules are stabilized by a pair of β sheets in the ATP binding domain and by an α helix in the catalytic domain. (*d*) Space-filling model of heterodimer of EGFR (red) and p185$^{c\text{-}neu}$ (blue). In the heterodimer, the intermolecular interactions are different than those of the homodimers, and based on energetic, heterodimer formation is more stable than homodimers. The autophosphorylation tyrosines (pink) are poised in such a fashion that, in heterodimers, these tyrosines might act like a gate to modulate and control signal transduction by altering structure and selection of substrate (see text for details).

crystal structures of kinases. In the catalytic region of both p185$^{c\text{-}neu}$ and EGFR, the Lys-Pro-Glu of cAPK has been substituted by Ala-Ala-Arg, a feature suggested to be characteristic of the tyrosine kinase family proteins (29). The structural features of the ATP binding domain is conserved despite the fact residues Lys-168 and Glu-170 in cAPK are changed to alanine and arginine in both p185$^{c\text{-}neu}$ and EGFR. The arginine residue in p185$^{c\text{-}neu}$ and EGFR may play a different role in stabilizing charge distribution in the substrate binding domain.

Nonconserved residues in the kinase domains of p185$^{c\text{-}neu}$ and EGFR are located near the activation loop and near the surface of the molecule. The most variable region, the activation loop, is distinct in both p185$^{c\text{-}neu}$ and the EGFR and remains flexible in both (Fig. 2). We consider the activation loop as perhaps the most defining region of a particular kinase. The activation loop contains some of sites for autophosphorylation. In the case of EGFR, the Tyr-845 is partially oriented toward the active site, whereas in p185$^{c\text{-}neu}$, Tyr-882 appears to

Table 1. Results of energy and surface calculations for oligomers of the kinase domains of p185$^{c\text{-}neu}$ and EGFR

Surface accessibility	neu–neu	EGFR–EGFR	neu-EGFR	Tetramer*
Buried, Å^2	1653	1553	2536	5670
Hydrophilic	458 (28%)	477 (31%)	581 (23%)	1481 (26%)
Hydrophobic	1195 (72%)	1075 (69%)	1955 (77%)	4189 (74%)
Energy, kcal/mol				
VDW contribution	−2870	−2560	−3201	−414
Electrostatic	−30874	−29686	−31028	−47113

Surface accessibility and intermolecular energy were calculated by using QUANTA (Molecular Simulations) with a probe radius of 1.4 Å.
*Calculations were performed by using XPLOR (19).

FIG. 4. Surface model of heterodimer of the kinase domains of p185$^{c\text{-}neu}$ and EGFR. Blue color represents the distribution of positive charges, and red represents the negative charges. The surface charge distribution shows separation of charge clusters. A dipole induced by the charge separation might initiate oligomerization when kinase domains approach within close proximity and also act as a stabilizing force.

be disposed on the surface. This feature was rather unexpected given the observation that in the crystal structures of cAPK and IRK, the equivalent self-phosphorylation sites oriented toward the active site. The environment of the Tyr-882, the autophosphorylation site, is more negative in p185$^{c\text{-}neu}$ than the autophosphorylation site of cAPK or IRK. In the activation loop, the autophosphorylation sites in p185$^{c\text{-}neu}$, EGFR, and cAPK are preceded by an amino acid sequence Asp-Glu-Thr-Glu, Glu-Glu-Lys-Glu, and Gly-Arg-Thr-Trp, respectively (Fig. 1). Lack of a positive charge near the autophosphorylation site leaves the activation loop in p185$^{c\text{-}neu}$ negative, and this feature disposes Tyr-882 on the surface pointing toward the active site. The IRK activation loop has been observed to be very mobile as judged by high thermal factors (35). The mobility of the activation loop is consistent with large movement of this region observed in p185$^{c\text{-}neu}$ and EGFR during the short molecular dynamics calculation we have undertaken. During the dynamics calculation, we observed that some autophosphorylation tyrosines could fold inwards for cis-phosphorylation. The positively charged residue preceding Tyr-845 in the EGFR is attracted toward the negatively charged catalytic site, favoring the activation loop to fold inwards. No such force was exerted on the activation loop of p185$^{c\text{-}neu}$ to fold inwards. This results in the autophosphorylation site of EGFR (Tyr-845) being partially buried, while the autophosphorylation tyrosine Tyr-882 of p185$^{c\text{-}neu}$ appears on the surface. In ERK-2, one of the autophosphorylation sites, Thr-183, preceded by uncharged residues, is also observed on the surface and is phosphorylated.

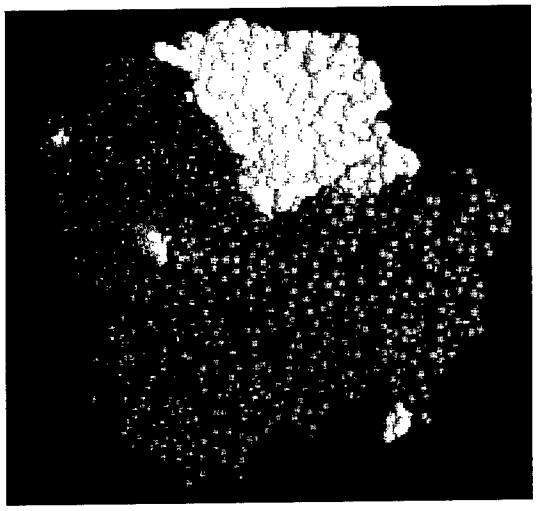

FIG. 5. Space-filling model of tetrameric association of the kinase domains of p185$^{c\text{-}neu}$ and EGFR. The tetramer is viewed down the pseudo-4-fold axis, tilted toward the viewer. The autophosphorylation tyrosines of p185$^{c\text{-}neu}$ and EGFR are more involved in tetrameric structural integrity, where any change, such as phosphorylation or mutation, might induce an allosteric-related structural change.

The substrate binding pocket in dimers of p185$^{c\text{-}neu}$ and EGFR is wide near the substrate binding region of the monomer, narrow near the active sites, and wide again at the back. Although the substrate binding pocket in dimers has similar shape, the dimension of the pocket is different. Both the p185$^{c\text{-}neu}$ homodimer and the heterodimer have smaller binding pockets compared to the binding pocket of EGFR. The substrate binding pocket of the EGFR homodimers is widened by the presence of Tyr-891 located near the interface. The difference in the size of binding pockets among the dimers suggests that the dimers might be used to recruit different substrates of different sizes in a structure dependent manner.

Jones and Thornton (36), in a recent survey of protein–protein interactions of dimers, suggest that the amino acid composition at the interface of dimers can be hydrophilic, resembling the surface of a protein. Nevertheless, the interface is stabilized by hydrophobic interactions rather than hydrophilic interactions. In general, the protein dimers are stabilized by an average of 8 hydrogen bonds per 1000 Å^2 buried surface (36). Results from our analysis of p185$^{c\text{-}neu}$–EGFR dimers are consistent with their results. Our data also indicate that the interfaces of homodimers and heterodimers of p185$^{c\text{-}neu}$ and EGFR are equally rich in hydrophobic residues. The distribution of hydrophobic residues at the interface would increase the avidity to form dimers and energetically act like a glue (37). The dimeric surface of p185$^{c\text{-}neu}$ and EGFR is stabilized by an average of 15 hydrogen bonds. The average area of surface lost on dimer formation of p185$^{c\text{-}neu}$ and EGFR is 1696 Å, and it is contributed by 68.1% of nonpolar atoms and 21.9% by polar atoms. These values are also in the range with that observed for other protein dimers surveyed by Jones and Thornton (36).

Results from the conformational energy calculations indicate that heterodimers would be preferred over homodimers. In addition, the larger area of buried surface in forming heterodimers over homodimers suggests that the heteromer would be more stable than the homomer. Thus, if a cell expresses both p185$^{c\text{-}neu}$ and EGFR, then heterodimer formation would be favored over homodimer formation. This is in agreement with our biochemical observations (14) that coexpression of truncated p185$^{c\text{-}neu}$ and full length EGFR results in a predominant intermediate size heterodimer over either p185$^{c\text{-}neu}$ or an EGFR homodimer.

Fig. 4 shows the surface charge distribution in the heterodimer. The core is negatively charged, and one end of the molecule contains a positively charged cluster. The positive charge spans from one side of EGFR to the interface of the p185$^{c\text{-}neu}$. A similar charge distribution is observed in the homodimers. The separation of charges suggests that the dimers might be stabilized by a net dipole–dipole interaction when the two kinase domains are juxtaposed. Thus, the distance within which the kinase domains must be brought into juxtaposition would be critical. In support of this concept, bivalent monoclonal antibodies have been shown to induce dimerization where as the Fab fragment derived from the monoclonal alone failed to do so (38). It is structurally possible that the antibody, with two Fab fragments disposed apart at least by about 36 Å, can bind to two receptors and bring them within a distance that might induce a favorable dipole–dipole interaction between the kinase domains.

It is known that autophosphorylation plays a crucial role in signal transduction (39). Various receptors have a tendency to phosphorylate both cis and trans on either Ser/Thr or Tyr. In the case of $p185^{c\text{-}neu}$ and EGFR, most of the autophosphorylation sites are located outside of the kinase domain, at the C terminus of the receptor. The complete role of the autophosphorylation sites in the kinase domain is not known definitively, but autophosphorylated tyrosines have been implicated as binding site for Src homology 2 domain-containing substrates (44). The autophosphorylation sites Tyr-882 in $p185^{c\text{-}neu}$ and Tyr-845 in EGFR are located in the activation loop and near the interface of the dimer. The orientation of these tyrosine are very different in $p185^{c\text{-}neu}$ and EGFR, although they are located at the same place within the activation loop. In the $p185^{c\text{-}neu}$ homodimer, the autophorylation tyrosine (Tyr-882) is pointing outwards (Fig. 3*a*), suggesting that this tyrosine may not be involved in the cis-phosphorylation and would not affect kinase activity. In contrast, the autophosphorylation tyrosine in EGFR (Tyr-845) is partially buried (Fig. 3*b*), suggesting that the tyrosine can undergo cis-autophosphorylation. These tyrosines in EGFR are located about 15 Å away from the interface of the dimer and oriented toward the substrate binding region, such that they could influence substrate binding. Thus, the model suggests that in the case of $p185^{c\text{-}neu}$, that autophosphorylation of Tyr-882 might not be critical to kinase activity. However, this residue may be phosphorylated by other proteins, provided it is not buried by the C-terminal residues. Thus, the location and orientation of these tyrosines implies that they play a critical role in the selection of substrates in various protein aggregation states.

The C terminus of the cytoplasmic domain is not conserved within the tyrosine kinase family receptors (40). Lack of sequence homology limits modeling of this fragment. In the crystal structure of cAPK (18) and twitchin kinase (34), the C terminus (regions outside the kinase domain) folds back close to the active site of the enzyme. It is possible that nonhomologous proteins within a family will have similar structures (41). Based on such observations, the C terminus of both $p185^{c\text{-}neu}$ and EGFR might fold back and approximate to the active site.

EGFR and $p185^{c\text{-}neu}$ may also undergo higher orders of aggregation (42). Yarden and Schlessinger (38) have demonstrated that noncovalent forces appear relevant to EGFR oligomerization using nondenaturing gel analysis. Lax *et al.* (43) have shown that EGFR can undergo higher order of oligomerization upon binding to EGF. Formation of dimers and tetramers have been deduced from electron microscopic analysis. Assuming the aggregation is ordered and specific for dimers, the next higher order of aggregation would be a tetramer. Model building shows that such a possibility exists. The substrate binding region in the tetrameric form is very similar to that of the dimers. However, the interactions among the subunits of $p185^{c\text{-}neu}$ and EGFR are very different. In a tetramer, the role of these tyrosines may even involve structural changes induced by an allosteric effect (Fig. 5). One of the important consequences is that the autophosphorylation tyrosines are positioned at the interface like a gate, resulting in less freedom of movement among the subunits. Thus, it is possible that in a cell expressing both $p185^{c\text{-}neu}$ and EGFR, dimers would be formed initially, and over time the dimers could associate as tetramers. This transition, then, might be used to regulate signals and to provide unique scaffolds for adaptor molecules

Our modeling investigated several features of homodimeric and heteromeric complexes of $p185^{c\text{-}neu}$ and EGFR kinase domains. Our study demonstrates the propensity of kinase domains to complex within a cell and features of the nature of these complexes. Consistent with observed biochemical data, our model confirms that heterodimers would be preferred over homodimers. Our models also suggest that the autophosphorylation tyrosine in the kinase domains might be involved in altering the oligomeric structure by allosteric mechanism to recruit different substrates without altering kinase activity. These models may facilitate the design and development of inhibitors of heteromeric kinase complexes that are relevant to neoplastic disease.

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