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Carboxyl-terminal deletion and point mutations decrease the transforming potential of the activated rat *neu* oncogene product

(protein-tyrosine kinase/site-directed mutagenesis/oncogene regulation)

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ABSTRACT **The rat *neu* oncogene encodes a constitutively activated growth factor receptor/transmembrane tyrosine kinase, p185Tneu, that is structurally similar to yet distinct from the epidermal growth factor receptor. To explore the role of the carboxyl-terminal region and of putative autophosphorylation sites in regulating the activity of the rat p185Tneu (T, transforming) protein, we used site-directed mutagenesis to generate a p185Tneu mutant in which a putative tyrosine autophosphorylation site (residue 1253) at the extreme carboxyl terminus was replaced by a phenylalanine residue and a mutant in which the carboxyl-terminal 122 amino acids were deleted. These proteins were expressed in NIH 3T3 cells at comparable levels and exhibited similar autophosphorylation activity, exogenous substrate phosphorylation ability, oligomerization levels, and responsiveness to a partially purified neu-activating factor. However, the mutant p185Tneu proteins displayed a decreased transforming capacity both *in vitro* and *in vivo*. This analysis demonstrated that the carboxyl-terminal domain and at least one putative tyrosine autophosphorylation site of p185Tneu play a role in positively regulating the cell growth-regulating properties of the neu protein.**

Transmembrane tyrosine kinases such as the neu protein, its human homologue the c-erbB-2 protein, and the closely related epidermal growth factor receptor (EGFR) possess a cysteine-rich extracellular ligand-binding domain, a hydrophobic membrane-spanning domain, and a cytoplasmic tyrosine kinase domain (1). Ligand binding to the extracellular domain results in receptor aggregation, activation of the kinase domain, and phosphorylation of intrinsic substrates (2). How these signals are ultimately translated into mitogenic and/or differentiation-altering effects is not fully known.

The rat *neu* oncogene product is termed p185Tneu (T, transforming) and was originally identified as the transforming protein in chemically induced rat neuroglioblastomas (3, 4). Constitutive activation of its tyrosine kinase domain was found to confer its potent transforming activity compared with its nontransforming cellular homologue, p185c-neu (5–7). The EGFR protein cannot transform cells in the absence of ligand (8). The oncogenic p185Tneu protein can, however, completely transform NIH 3T3 cells. Thus, in the absence of cloned, purified ligand for the neu protein, the p185Tneu protein represents a system in which elevated kinase activity and transforming ability (both reminiscent of ligand-induced activation) are already present.

Though EGFR and the *neu*/c-*erbB-2* gene product share extensive structural and sequence homology, these proteins are only 30% homologous in their carboxyl-terminal region (9, 10). Their dissimilar carboxyl-terminal domains, which contain autophosphorylation and substrate association sites, most likely contribute to the apparent differential regulation of these closely related transmembrane tyrosine kinases (11, 12). The role of the carboxyl-terminal region and of the autophosphorylation sites contained within this region have been explored as they relate to EGFR and c-erbB-2 regulation but have not been examined extensively for rat neu protein regulation.

To explore the role of the carboxyl-terminal region and of putative autophosphorylation sites in the regulation of the rat neu protein, we have generated mutants of the carboxyl-terminal region of p185Tneu. The constructs encoding these mutants were transfected into NIH 3T3 mouse fibroblasts for subsequent phenotypic characterization. Studies of these mutant proteins and of the wild-type p185Tneu protein in the same cellular backround at comparable expression levels permitted the investigation of the role of the carboxyl-terminal region and of putative autophosphorylation sites in neu protein regulation.

MATERIALS AND METHODS

Construction of Mutants. Plasmid pSV2neuT contains a cDNA that encodes rat p185Tneu (9). A 2.7-kilobase DNA fragment (from pSV2neuT) spanning the carboxyl-terminal region of the p185Tneu protein was subcloned into the plasmid pGEM-5Zf (Promega). Single-stranded uridylated DNA template was generated (13). *In vitro* oligonucleotide-directed mutagenesis was performed (14) and the mutations were verified by DNA sequencing (15). The appropriate mutated fragments were isolated and ligated back into pSV2neuT to generate plasmids encoding p185Tneu mutants with a substitution of phenylalanine for tyrosine-1253 (pSV2T/1253F) or a deletion of 122 amino acids from the carboxyl terminus by insertion of a stop codon (pSV2T/APstop).

Preparation and Maintenance of Cell Lines. The constructs were cotransfected with pSV2neo into NIH 3T3 cells (which are devoid of endogenous EGFR) by calcium phosphate precipitation. After 3 weeks of G418 selection, the resultant colonies were checked for the binding of 7.16.4, a monoclonal antibody that recognizes the extracellular domain of p185c-neu and p185Tneu (16), by flow cytometry. The cells that expressed p185 were further cloned by the limiting dilution technique prior to phenotypic characterization. The NIH

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Abbreviations: EGFR, epidermal growth factor receptor; NAF, neu protein-activating factor; BS^3, bis(sulfosuccinimidyl) suberate.

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3T3/Tneu, NIH 3T3/T/1253F, and NIH 3T3/T/APstop cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum under an atmosphere of 5% CO_2 at 37°C and were passaged every 3–4 days.

Western Blotting. Immunoprecipitation and Western blotting were performed as described (17, 18). The antibodies used were an anti-p185 intracellular domain antibody (anti-Bacneu; J.N.M. and M.I.G., unpublished data), DBW-2 (19), or monoclonal anti-phosphotyrosine antibody PY-20 (ICN).

Chemical Crosslinking/Dimerization Assay. Cells (3×10^6) in 10-cm culture dishes were incubated in DMEM supplemented with 5% fetal bovine serum and washed twice with cold phosphate-buffered saline. Five milliliters of phosphate-buffered saline containing the bifunctional covalent, lipid-impermeant crosslinking reagent bis(sulfosuccinimidyl) suberate (BS^3, Pierce) at 4 mM was added and cells were incubated at 22°C for 30 min. The crosslinking reaction was quenched in 10 mM Tris, pH 7.5/0.15 M NaCl. Cells were solubilized with 1% (vol/vol) Nonidet P-40/1% (wt/vol) sodium deoxycholate/1% (vol/vol) Trasylol/1 mM phenylmethylsulfonyl fluoride/2 mM EDTA/10 mM iodoacetamide/1 mM ATP prior to SDS/PAGE of the crosslinked proteins in 4–7% gradient gels (18).

***In Vitro* Kinase Assay.** Preconfluent cultures of cells in 150-mm dishes were washed, and cell lysate was prepared (17). Aliquots of precleared cell lysate were incubated on ice for 30 min with the indicated concentration (percent by volume) of neu protein-activating factor (NAF) (20) and subjected to immunoprecipitation with 5 μg of antibody 7.16.4, followed by a 50-min incubation with 50 μl of 50% (vol/vol) protein A-Sepharose with rotation at 4°C. Immune complexes were collected as described (17). The samples were incubated on ice for 45 min. The reactions were terminated by heating at 100°C for 5 min. The samples were then subjected to SDS/14% PAGE prior to autoradiography.

Down-Regulation Studies. These studies were performed as described (21). Cells (10^5) of each cell line were seeded in 24-well dishes (Costar) and incubated overnight in DMEM supplemented with 5% fetal bovine serum. The cells were then incubated with DMEM supplemented with insulin, transferrin, and selenium (ITS; Collaborative Research) for 24 hr, washed, and incubated 1 hr at 37°C with binding buffer [DMEM with 20 mM Hepes (pH 7.2) and 0.1% bovine serum albumin]. Cells were then incubated with 10% NAF for the indicated time. The monolayer was washed with cold binding buffer and incubated at 0°C with or without 7.16.4 (2 μg/ml) in ITS/DMEM for 2 hr. Antibody solution was aspirated and the cells were incubated with 200 μl of ice-cold binding buffer with a 1:100 dilution of ^{125}I-labeled protein A (15 μCi/μg, NEN; 1 μCi = 37 kBq) for 45 min at 0°C. Cells were washed extensively with cold binding buffer and solubilized in 1 M NaOH for γ counting. Specifically bound radioactivity (cpm) was calculated by subtracting the cpm bound in the absence of primary antibody from the total cpm bound. All experiments were completed in duplicate.

Focus Formation Assay. One hundred cells from each cell line were coplated in 6-cm culture dishes with 10^4 NIH 3T3 cells and cultured in the DMEM containing 5% fetal bovine serum. The medium was changed every 3–4 days. On day 21 the plates of cells were fixed with 10% formalin and then stained with hematoxylin to observe morphologically transformed foci.

Tumor Inoculation and Measurement. Cells (10^6) of each cell line were suspended in 0.1 ml of phosphate-buffered saline and injected subcutaneously in the mid-dorsum of NCR nude mice. Growing tumors were measured with vernier calipers every 3 days beginning with tumor appearance. Tumor volume was calculated as the product of tumor length, width, and vertical height. Student's *t* test was used to determine the significance between the groups on the fourth week.

RESULTS

Characterization of p185Tneu and Mutants. The structural organization of the native and mutant p185Tneu proteins is depicted in Fig. 1. The carboxyl-terminal region and several

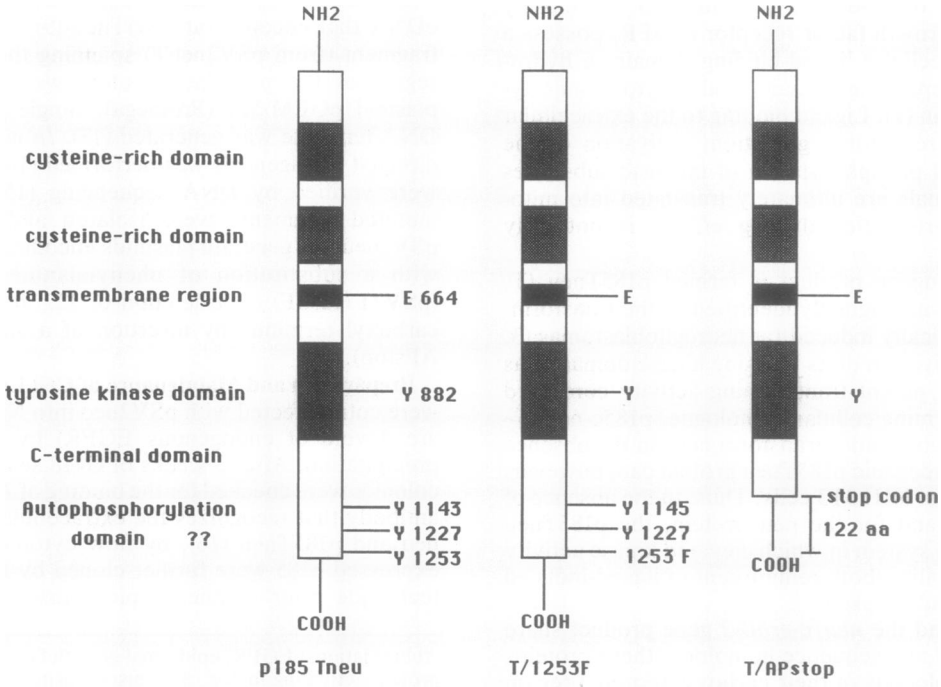

FIG. 1. Structure of p185Tneu and mutants p185T/1253F and p185T/APstop. The domain depicted above the transmembrane region represents the extracellular, amino-terminal domain. The domain below the transmembrane region represents the intracellular, carboxyl-terminal domain. Also shown are several putative tyrosine (Y) autophosphorylation sites that may be important in neu protein function. E, glutamic acid; F, phenylalanine; aa, amino acids.

putative tyrosine autophosphorylation sites are shown. To verify cell surface localization and examine expression levels, lysates from cell lines that displayed comparable levels of p185 protein (as determined by flow cytometry) were analyzed by immunoprecipitation with monoclonal anti-p185neu antibody 7.16.4 and Western blotting with either the intracellular domain-specific antibody anti-Bacneu, the anti-p185 intracellular domain peptide antiserum DBW-2 [against a synthetic peptide corresponding to amino acids 1240–1255 of p185neu (19)], or the anti-phosphotyrosine antibody PY-20 (Fig. 2A). All three proteins were expressed at similar levels, were recognized by anti-Bacneu, and possessed comparable levels of phosphotyrosine. The p185T/APstop protein was not recognized by DBW-2, because this antiserum was specific for carboxyl-terminal residues that have been deleted in the p185T/APstop mutant. These experiments indicated that the different forms of p185Tneu were expressed at similar levels and were appropriately processed and localized to the cell surface. Thus any differences between the cell lines can be attributed to the introduced mutations in the p185Tneu protein.

Dimerization/Oligomerization. Oncogenic p185Tneu exists in both monomeric and homodimeric forms. To assess the aggregation state of the p185Tneu proteins, covalent crosslinking studies were performed with the lipid-impermeant covalent crosslinking reagent BS^3. The crosslinked products (Fig. 2B) from each cell line had molecular weights comparable to that of p185Tneu homodimer from B104-1-1 cells (22). Anti-phosphotyrosine Western blots revealed that phosphotyrosine density was nearly equally distributed between the monomeric and dimeric forms of each p185Tneu species (Fig. 2B).

***In Vitro* Autophosphorylation and Phosphorylation of Exogenous Substrate.** The basal kinase activity of the mutant p185Tneu proteins was first assessed. Since the autophosphorylation and substrate phosphorylation capabilities of the constitutively activated p185Tneu protein are higher than those of the p185c-neu, we included a cell line that expresses p185c-neu as a control and for comparison. As expected, basal kinase activity levels of native and both mutant p185Tneu proteins were higher than that of the p185c-neu protein (Fig. 3). Both mutant p185Tneu proteins displayed comparable basal autophosphorylation levels and exogenous substrate (histone H3) phosphorylation levels when compared with the native p185Tneu expressed in the same cellular background (Fig. 3).

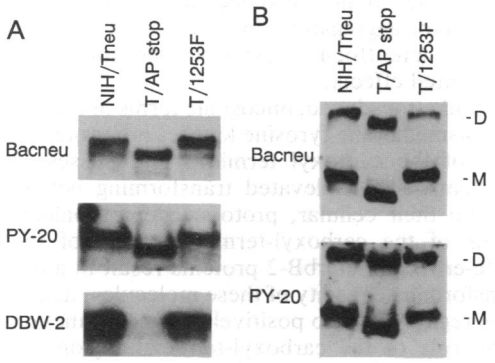

FIG. 2. Western blot and crosslinking analyses of p185Tneu and mutants. (A) Expression of p185Tneu and its mutants and assessment of their phosphotyrosine content in the transfected NIH 3T3 cell lines. Intact cells were lysed after overnight incubation in DMEM with 5% fetal bovine serum and subjected to immunoprecipitation with monoclonal anti-p185 antibody 7.16.4. Proteins were separated by SDS/8% PAGE, transferred to nitrocellulose, and probed with anti-p185 intracellular domain antibody (Bacneu), or with monoclonal anti-phosphotyrosine antibody (PY-20), or with a polyclonal antiserum against the carboxyl-terminal region of p185 (DBW-2). Antibody binding was visualized with iodinated protein A and autoradiography. (B) Monomer/dimer stoichiometry of surface p185Tneu and its mutants. Each cell line (3×10^6) was incubated in DMEM with 5% fetal bovine serum overnight at 37°C and treated with BS^3 at 22°C. Lysates were immunoprecipitated using 7.16.4. Proteins were separated by SDS/PAGE in a gradient gel and transferred to nitrocellulose. Monomeric (M) and homodimeric (D) forms of p185Tneu and mutants were detected with anti-p185 antibody (Bacneu) and monoclonal anti-phosphotyrosine antibody (PY-20).

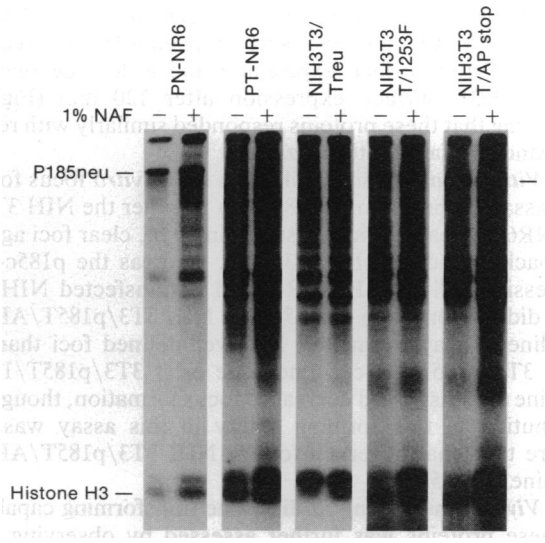

FIG. 3. *In vitro* immune-complex autophosphorylation and exogenous substrate phosphorylation by p185Tneu and its mutants with (+) or without (−) 1% NAF (by volume). p185 was immunoprecipitated from cell lysates and subjected to the *in vitro* kinase assay. Immune complexes were resuspended in a reaction mixture containing labeled ATP and the exogenous substrate histone H3 for 45 min on ice, prior to SDS/PAGE analysis. Cell lines are indicated above lanes.

Responsiveness to NAF was also examined. NAF interacts with the extracellular domain of the p185c-neu protein, resulting in neu protein kinase activation, dimerization, internalization, and growth potentiation (20). NAF-dependent enhancement of autophosphorylation and phosphorylation of the exogenous substrate histone H3 was found to be comparable for both mutants and for the wild-type p185Tneu protein (Fig. 3).

NAF-Dependent Internalization. To further assess the functioning of these proteins, we examined their ability to be internalized in response to NAF. Native p185Tneu surface

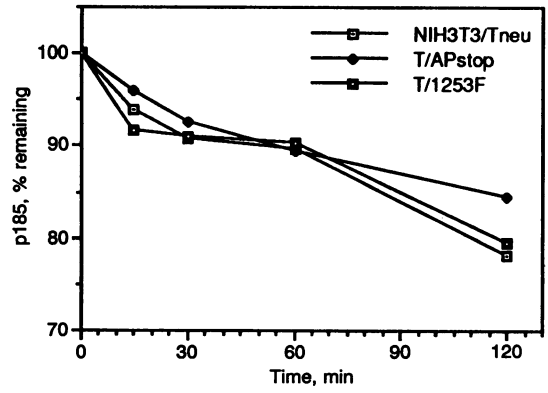

FIG. 4. Effect of NAF on internalization of p185Tneu and its mutants. Cells (5×10^4) were incubated with 10% NAF (by volume) for the indicated time at 37°C. After incubation, the amount of surface p185 was determined as described in *Materials and Methods*.

expression was decreased 10% at 30 min and 23% at 120 min (Fig. 4). With the mutants, NAF induced a 16% decrease in p185T/APstop surface expression and a 20% decrease in p185T/1253F surface expression after 120 min (Fig. 4), indicating that these proteins responded similarly with regard to ligand-mediated internalization.

***In Vitro* Transforming Ability.** In the *in vitro* focus formation assay, expression of p185Tneu in either the NIH 3T3 or the NR6 cell background resulted in large, clear foci against the background of NIH 3T3 cells, whereas the p185c-neu-expressing cell line (PN-NR6) and untransfected NIH 3T3 cells did not form foci (Fig. 5). The NIH 3T3/p185T/APstop cell line displayed smaller, less well-defined foci than the NIH 3T3/p185Tneu cell line. The NIH 3T3/p185T/1253F cell line also displayed decreased focus formation, though the diminution in transforming ability in this assay was less severe than that observed for the NIH 3T3/p185T/APstop cell line (Fig. 5).

***In Vivo* Transforming Ability.** The transforming capability of these proteins was further assessed by observing their ability to form progressive tumors in nude mice. Tumor volume at the end of the fourth week following tumor implantation was significantly larger for NIH 3T3/p185Tneu cells than for NIH 3T3/p185T/APstop cells, whereas the NIH 3T3/p185T/1253F cell line displayed an intermediate phenotype (Fig. 6).

DISCUSSION

The oncogenic, deregulated p185Tneu protein was used to define determinants involved in regulating the transforming ability of the activated neu protein. Site-directed mutagenesis was employed to delete or alter regions in the carboxyl-terminal region thought to be involved in p185Tneu regulation. In the mutant designated p185T/1253F, a putative autophosphorylation site, tyrosine-1253 of p185Tneu, was changed to phenylalanine. In the second p185T/APstop, the carboxyl-terminal 122 amino acids (including three putative tyrosine autophosphorylation sites) were deleted. The mutant proteins displayed comparable autophosphorylation and exogenous substrate phosphorylation activity, receptor aggregation state, and ability to respond to NAF when compared with native p185Tneu. However, both of the mutants displayed decreased transforming ability *in vitro* as well as *in vivo*. The ability of p185T/APstop to induce foci *in vitro* and tumors in nude mice was impaired and p185T/1253F protein was somewhat impaired. The decreased transforming ability of the p185Tneu/APstop truncation mutant and the p185T/1253F substitution mutant implicates the carboxyl terminus itself and at least one putative tyrosine autophosphorylation site within this region in the positive regulation of the rat neu protein. These studies also indicate distinctions between early (receptor aggregation, internalization, and phosphorylation) and late (focus formation and tumor formation) growth-related effects.

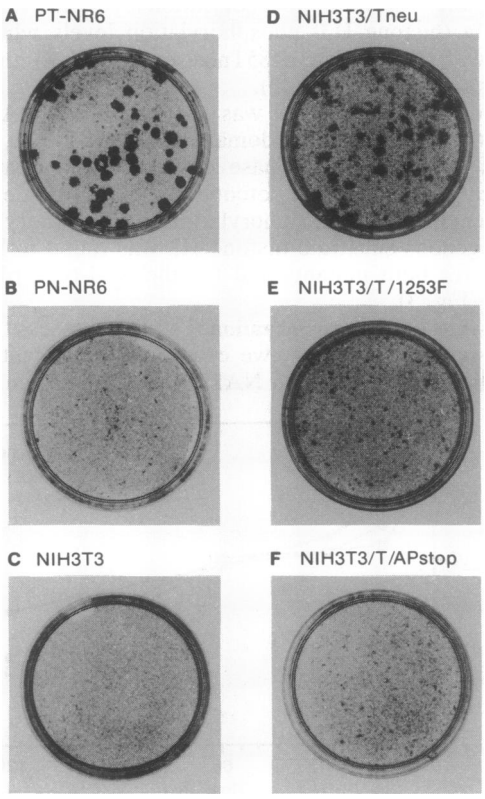

FIG. 5. Focus formation assay of p185Tneu and its mutants. One hundred cells of each cell line were cocultured with 10^4 NIH 3T3 cells in DMEM with 5% fetal bovine serum. Cells were fixed with 10% formalin and stained with hematoxylin on day 21.

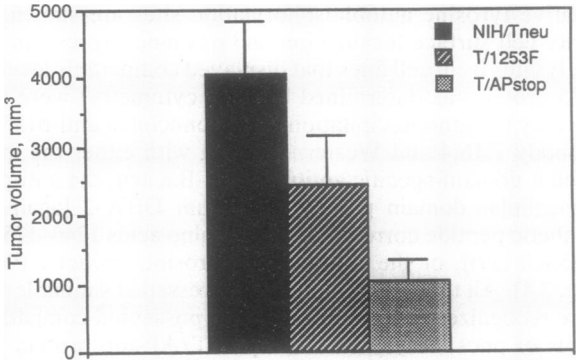

FIG. 6. Tumor formation in nude mice. Transfected cells (10^6) expressing native and mutant p185Tneu proteins were suspended in 0.1 ml of phosphate-buffered saline and injected subcutaneously in the mid-dorsum of NCR nude mice. Growing tumors were measured and statistical analysis was performed. Vertical bars indicate SEM.

The virally transduced, oncogenic forms of the c-erb-B and c-fms transmembrane tyrosine kinases are proteins that lack portions of the carboxyl terminus and possess activated kinase domains and elevated transforming potential compared with their cellular, protooncogenic homologues (1). Deletions of the carboxyl-terminal region of the human EGFR/c-erbB and c-erbB-2 proteins result in a decrease in the transforming capacity of these molecules, demonstrating that this region can also positively regulate function (11, 23, 24). The role of the carboxyl-terminal region of the neu protein suggested by our studies is consistent with these studies. However, other studies have described contradictory results. One recent study (25) described a carboxyl-terminally truncated c-erbB-2 mutant that had a 3-fold increase in *in vitro* focus formation relative to the full-length protein containing the transforming transmembrane point mutation. Another study (26) found no difference in the transforming ability of a rat p185Tneu mutant that lacked most (≈270 amino acids) of the carboxyl-terminal region. The differences between these data on the role of the carboxyl-terminal region in transformation may be due to differences between the human and rat neu protein sequences, to differences in their regulation, or to the different sizes of the deletions used in each study. In addition, some studies examined only one characteristic of the malignant phenotype (i.e., focus formation or growth rate) and did not examine *in vivo* tumorigenicity.

Autophosphorylation may play a different role in regulating the activity of different tyrosine kinases. Though auto-

phosphorylation has been shown to alter the activity of other tyrosine kinases such as the c-*src* gene product, the insulin receptor, and the c-*fms* gene product (27), studies of the consequences of EGFR autophosphorylation have given conflicting results. Some studies have shown that EGFR autophosphorylation increased the activity of receptor kinase (28), while others, using comparable assays, have found little change in the enzymatic or biological activity of this protein (29–31). The role of autophosphorylation in the regulation of the neu protein or its human homologue, the c-erbB-2 protein, has not been adequately addressed, although some of the potential autophosphorylation sites of the human c-erbB-2 protein have been mapped (32, 33).

We examined the role of a putative autophosphorylation site of the constitutively activated p185Tneu protein expressed by a simian virus 40 promoter/expression system and detected a significant, positive role of this autophosphorylation site in transformation. The alteration of the transforming capacity of the neu protein as a result of the site-directed mutagenesis of a single autophosphorylation site (tyrosine-1253) was not fully recognized in previous studies. Human c-erbB-2 autophosphorylation-site mutants did not reveal a role for any individual autophosphorylation site in *in vitro* transformation assays (24). This study was complicated by the extremely high levels of c-erbB-2 protein expression driven by a Moloney leukemia virus long terminal repeat promoter. These levels of receptor expression may result in less stringent requirement for the phosphorylation of each individual tyrosine residue for transformation. However, multiple autophosphorylation-site mutations did result in a decrease in the transforming potential of the c-erbB-2 protein (24), which is consistent with our own findings. Another recent study of c-erbB-2 autophosphorylation states revealed a decrease in the transforming ability when tyrosine-1248 was changed to phenylalanine (25). This finding is similar to ours, since the autophosphorylation-site mutation was introduced into a c-erbB-2 protein that also contained a substitution of valine for glutamate-659, and we introduced our autophosphorylation-site mutation into the rat neu protein, which also possesses this transmembrane-region mutation.

We have presented evidence that the availability of tyrosine-1253 and of other autophosphorylation sites (contained within the 122-amino acid deletion) may be critical in positively regulating some of the growth/transforming abilities of p185Tneu. In addition, the magnitude of the decrease in transforming potential correlated with the number of tyrosine autophosphorylation sites that were deleted. Therefore, some of the differences in the neu and EGFR signaling potential revealed through studies of receptor chimeras (11) and some of the differences in their signal-coupling efficacy in different cell types (12) could be related to differential consequences of autophosphorylation between these two receptors. Future studies of the role of autophosphorylation and of the carboxyl-terminal region as a whole in neu protein regulation, particularly in the p185c-neu context, will be facilitated by a more complete understanding of the substrate specificities of this protein and by the complete purification and availability of its ligand.

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