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An Oncogenic Point Mutation Confers High Affinity Ligand Binding to the *neu* Receptor

IMPLICATIONS FOR THE GENERATION OF SITE HETEROGENEITY*

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The *neu* protooncogene encodes a receptor tyrosine kinase homologous to the receptor for the epidermal growth factor. The oncogenic potential of *neu* is released upon chemical carcinogenesis, which replaces a glutamic acid for a valine residue, within the single transmembrane domain. This results in constitutive receptor dimerization and activation of the intrinsic catalytic function. To study the implications of the oncogenic mutation and the consequent receptor dimerization on the interaction with the yet incompletely characterized ligand of p185^{neu}, we constructed chimeric proteins between the ligand binding domain of the epidermal growth factor receptor and the transmembrane and cytoplasmic domains of the normal or the transforming Neu proteins. The chimeric receptors displayed cellular and biochemical differences characteristic of the normal and the transforming Neu proteins and therefore may reliably represent the ligand binding functions of the two receptor forms. Analyses of ligand binding revealed qualitative and quantitative differences that were a result of the single mutation; whereas the normal chimera (valine version) displayed two populations of binding sites with ~90% of the receptors in the low affinity state, the transforming receptor (glutamic acid version) showed a single population of binding sites with relatively high affinity. Kinetics measurements indicated that the difference in affinities was because of slower rates of both ligand association and ligand dissociation from the constitutively dimerized mutant receptor. It therefore appears that the oncogenic mutation, by permanently dimerizing the receptor, establishes a high affinity ligand binding state which is functionally equivalent to the ligand-occupied normal receptor. Our conclusion is further supported by the rates of endocytosis of the wild-type and the mutant receptor. Hence, these results provide the first experimental evidence from living cells which supports a model that attributes the heterogeneity of ligand binding sites to the state of oligomerization of receptor tyrosine kinases.

The *neu* protooncogene (also called HER-2 and c-*erbB*-2) encodes a 185-kDa transmembrane glycoprotein that is highly homologous to but yet distinct from the epidermal growth factor (EGF)[1] receptor (Coussens *et al.*, 1985; Yamamoto *et al.*, 1986; Bargmann *et al.*, 1986a). The oncogenic potential of the rat *neu* gene was found to be activated by a carcinogen-induced point mutation that replaces a valine residue within the single transmembrane domain for a glutamic acid residue (Bargmann *et al.*, 1986b). The transmembrane stretch, as in all receptor tyrosine kinases (Yarden and Kelman, 1991), connects the ligand binding domain with the catalytic tyrosine kinase domain that faces the cytoplasm. The oncogenic mutation was found to affect dramatically the biochemical properties of the presumed receptor: the intrinsic tyrosine kinase function was permanently stimulated both *in vitro* (Bargmann and Weinberg, 1988a; Segatto *et al.*, 1988) and in living cells (Yarden, 1990), and the rate of turnover of the mutant receptor was markedly accelerated (Stern *et al.*, 1988; Yarden, 1990). These biochemical characteristics can be conferred to the normal gene product by a monoclonal antibody but not by a monovalent Fab fragment of it, indicating a role for receptor dimerization in kinase activation (Yarden, 1990). Indeed, theoretical (Sternberg and Gullick, 1990; Brandt-Rauf *et al.*, 1990) and experimental evidence (Weiner *et al.*, 1989) implied that the transforming mutation permanently maintains the receptor in a dimer form that is analogous to the ligand-induced dimer of the EGF receptor (Yarden and Schlessinger, 1987a, 1987b).

The implications of the oncogenic mutation of p185^{neu} for the interaction with its presumed ligand molecule are yet unknown. The existence of a ligand molecule of p185^{neu} was originally inferred on the basis of bioassays which detected the putative ligand in medium conditioned by *ras*-transformed fibroblasts (Yarden and Weinberg, 1989). This activity was later purified and found to correspond to a 30-35-kDa glycoprotein (Yarden and Peles, 1991). An apparently similar activity is secreted by human breast cancer cells (Lupu *et al.*, 1990) and by transformed human T-cells (Dobashi *et al.*, 1991). Despite these initial observations the ligand molecule is not yet available for direct binding studies. To overcome it, we and others employed a chimeric receptor approach whereby the extracellular domain of p185^{neu} was replaced by the ligand binding domain of the receptor for EGF (Lee *et al.*, 1989; Lehvaslaiho *et al.*, 1989; Peles *et al.*, 1991). This configuration allows heterologous stimulation of the *neu*-encoded tyrosine kinase and has been successfully exploited in signal

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[1] The abbreviations used are: EGF, epidermal growth factor; EDAC-1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid); PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Ab, antibody; NCT, Neu carboxyl terminus.

transduction studies (Sistonen *et al.*, 1989; Peles *et al.*, 1991; Fazioli *et al.*, 1991).

The interactions of many different polypeptide ligands with their receptors display elevated binding affinity at low ligand concentrations. This phenomenon is commonly demonstrated by curvilinear Scatchard curves (Scatchard, 1949). Although heterogeneity of the ligand molecules could be involved in nonlinear Scatchard plots (Taylor, 1975), the existence of more than a single population of binding sites, or alternatively cooperative binding, was postulated in most cases (for review see Carpenter, 1987). In contrast with the receptor for nerve growth factor (Sutter *et al.*, 1979) and some lymphokine receptors (reviewed in Nicola and Metcalf, 1991), which have two binding affinities that differ by 2 orders of magnitude, other receptors display closer affinities (usually 20-fold difference; for review see Carpenter, 1987). Unlike lymphokine receptors, in which heterotypic molecular interactions generate the high affinity state (Wang and Smith, 1987), homodimers of the EGF receptor appear to possess higher ligand affinity as compared with monomeric receptors (Yarden and Schlessinger, 1987a, 1987b; Boni-Schnetzler and Pilch, 1987). Moreover, heterodimers of the EGF receptor and the Neu protein are also characterized by high affinity ligand binding (Goldman *et al.*, 1990; Wada *et al.*, 1990). However, both kinase activation and the interconvertibility of high and low affinity sites for EGF can occur with no involvement of receptor dimerization (Koland and Cerione, 1988; Northwood and Davis, 1988; Verheijden *et al.*, 1991). These observations do not favor the simple model in which receptor dimers represent the catalytically active high affinity population, and an alternative model involving phosphorylation of other membrane proteins was postulated (Walker and Burgess, 1991). The major problem in approaching this question is the strict dependence of receptor dimerization on the presence of the ligand. The possibility that the oncogenic transmembrane mutation of $p185^{neu}$ autonomously creates receptor dimers (Weiner *et al.*, 1989; Sternberg and Gullick, 1990; Yarden, 1990; Brandt-Rauf *et al.*, 1990) thus enables direct examination of the relationships between binding affinity and receptor oligomerization. By employing the chimeric Neu-EGF receptor proteins here we present results which support the possibility that dimerization is involved in the generation of high affinity and catalytically active receptors.

EXPERIMENTAL PROCEDURES

Materials—EGF was supplied by Biomakor (Rehovot, Israel) and by Toyobo (New York). Radioactive materials were purchased from Amersham (Buckinghamshire, UK). Protein A coupled to Sepharose was obtained from Pharmacia (Uppsala, Sweden) or prepared in our laboratory. Molecular weight standards for gel electrophoresis were from Bio-Rad. All other chemicals were purchased from Sigma unless otherwise stated.

Cell Culture—The following previously described cell lines were used: DHFR-G8 cells which overexpress the wild-type $p185^{neu}$, B104-1-1 fibroblasts which overexpress the transforming mutant of $p185^{neu}$, and a *ras*-transformant of the DHFR-G8 cells (*ras*-G8) (Bargmann *et al.*, 1986b; Bargmann and Weinberg, 1988b). All cell lines were grown in Dulbecco's modified Eagle's medium containing 10% calf serum (from HyClone Laboratories, Logan, UT). For most experiments the monolayers were grown on fibronectin-coated dishes (10 μg/ml fibronectin in phosphate-buffered saline, PBS).

Buffered Solutions—HNTG buffer contained 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% (w/v) Triton X-100, and 10% glycerol. Solubilization buffer contained 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% (w/v) Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 50 mM NaF, 2 mM sodium orthovanadate, 1 mM EGTA, aprotinin (0.15 trypsin inhibitor unit/ml), 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. PBS contained 137 mM NaCl, 2.7 mM KCl, 7.9 mM $NaHPO_4$, and 1 mM KH_2PO_4 (pH 7.2). High wash contained 50 mM HEPES (pH 7.5), 500 mM NaCl, 0.1% SDS, 0.2% (w/v) Triton X-100, 5 mM EGTA, 20 mM NaF, 5 mM EDTA, and 2 mM NaV. Medium wash contained 50 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% SDS, 0.2% (w/v) Triton X-100, 5 mM EGTA, 20 mM NaF, 5 mM EDTA, and 2 mM NaV. Low wash contained 10 mM Tris (pH 8.0), 0.1% (w/v) Triton X-100, 20 mM NaF, 5 mM EDTA, and 2 mM NaV.

Antibodies—A polyclonal antiserum to phosphotyrosine (PT5) was generated as described by Kamps and Sefton (1988). The NCT antiserum was raised in rabbits injected with a synthetic peptide that corresponds to the 16 carboxyl-terminal amino acids of human $p185^{neu}$. The monoclonal antibody Ab528 to EGF receptor was described previously (Kawamoto *et al.*, 1983).

Construction of the NEC and TEC Expression Vectors and Establishment of Expressing Cell Line—To construct an EGF receptor-Neu chimeric DNA we used the previously described EGF receptor/*kit* chimeric DNA (Lev *et al.*, 1990) which encodes the whole extracellular domain of the EGF receptor fused to the human *kit* DNA. This DNA was cloned into pBluescript plasmid (Stratagene, La Jolla, CA) with its 3′ end flanking the *KpnI* site of the plasmid. A 600-base pair *BstEII-KpnI* DNA fragment that encodes most of the cytoplasmic portion of $p145^{kit}$ was removed and replaced with the double-stranded synthetic oligonucleotide 5′-GTCACCAGCCGGCCACATATGTCG-GTAC-3′; 3′-GTCGGCCGGTGTATACAGC-5′. Proper insertion of the oligonucleotide was verified by nucleotide sequence analysis. This oligonucleotide contained an internal *NaeI* site which was used to further delete a 1.5-kilobase *NaeI* DNA fragment that contained the rest of the *kit* DNA. The resulting intermediate plasmid, SK-EC, contained an *XhoI-NaeI* portion of the EGF receptor fused in frame to codons derived from the synthetic oligonucleotides designed to create a fusion segment with the unique *NdeI* site at the membrane-extracellular junction of the rat *neu* protein (Bargmann *et al.*, 1986a). A 2.0-kilobase *NdeI-KpnI* DNA fragment coding for the transmembrane and cytoplasmic portion of $p185^{neu}$ was cut out of a pBluescript vector and replaced, by ligation, a heptanucleotide *NdeI-KpnI* segment of the original synthetic oligonucleotide in the SK-EC plasmid, to yield SK-NEC. A similar *NdeI-KpnI* DNA fragment of the transforming *neu* gene was used in parallel to construct the SK-TEC plasmid. This DNA fragment differed from its normal homolog by a single base substitution which altered a valine residue to a glutamic acid residue in the predicted transmembrane domain of p185 (Bargmann *et al.*, 1986b). The fusion points of both NEC and TEC chimeric genes were sequenced to ensure integrity of the open reading frames. The resulting chimeric DNA pieces were cut out of SK-NEC and SK-TEC by using *SalI* and cloned into the compatible *XhoI* site of a mammalian expression vector downstream of the SV40 early promoter and the cytomegalovirus enhancer to yield the pCMV-NEC and pCMV-TEC expression vectors. NIH-3T3 cells (2.2 subline), which lack endogenous EGF receptor, were transfected by the calcium phosphate precipitation method with either pCMV-NEC or pCMV-TEC and a plasmid carrying the neomycin resistance gene. Following transfection a glycerol shock (15% glycerol in PBS for 2 min) was given after 4 h, and the cells were subcultured into medium containing 0.5 mg/ml G418 (GIBCO). Resistant colonies were individually grown and assayed for binding of radiolabeled EGF.

Foci Formation Assay—Rat-1 cells were plated at a concentration of 10^5 cells/90-mm plate. After 18 h the cells were transfected by the calcium phosphate precipitation method with 10 μg of plasmid DNA/plate. The medium was replaced every 4 days. Foci were visualized by Giemsa staining after 21 days.

Soft Agar Colony Formation Assay—The NEC and TEC cells were suspended with trypsin and plated at 10^4 cells/35-mm dish in the presence or absence of EGF (100 ng/ml) in Dulbecco's modified Eagle's medium containing 5% calf serum and 0.3% agar. The cultures were kept wet by adding fresh medium, and they were scored for colonies by photography after 20 days of culturing.

Biosynthetic Labeling with [^{35}S]Methionine and Immunoprecipitation—Subconfluent cells in 6-cm dishes were washed with methionine-free Dulbecco's modified Eagle's medium and grown for 16 h in the same medium supplemented with 10% dialyzed calf serum containing 50 μCi of [^{35}S]methionine/ml. The cells were washed three times with PBS and then scraped into 0.5 ml of solubilization buffer. The lysates were spun for 10 min at 4 °C in an Eppendorf centrifuge. Protein A-Sepharose (3 mg/sample) was suspended in 20 mM HEPES (pH 7.5) buffer, washed once, and incubated for 30 min at room temperature with monoclonal anti-EGF receptor antibody (Ab528). The protein A-Sepharose-antibody complex was washed three times with HNTG and incubated with the cell lysate for 90 min at 4 °C. The immunoprecipitates were washed twice with the high, medium,

and low salt buffers. Then, 3 volumes of sample buffer were added to washed immunoprecipitates, boiled for 5 min, and electrophoretically separated on SDS-polyacrylamide gels. For the determination of receptor turnover rates, the incubation with [^{35}S]methionine was followed by a variable length chase in fresh medium containing 10% calf serum with or without 100 ng/ml EGF. After the chase period the chimeric proteins were immunoprecipitated and the immunocomplexes separated by SDS-polyacrylamide gel electrophoresis. This was followed by autoradiography. The resulting autoradiograms were scanned using an automated densitometer to enable quantitation of the level of the 185-kDa protein band.

Covalent Cross-linking Experiments—Confluent monolayers of cells grown on 90-mm fibronectin-coated plates were washed with PBS and then incubated with or without 100 ng/ml EGF in PBS. After 10 min of incubation at 22 °C the cells were washed once with PBS; a cross-linking reagent, bis(succinimidyl succinate), was added to final concentration of 0.2 mM and the incubation continued for 1 h at 22 °C. Alternatively we used EDAC at 15 mM (Cochet *et al.*, 1988). The monolayers were then washed twice with PBS and lysed in solubilization buffer. Immunoprecipitations were performed as described above with either the NCT antiserum to p185^{neu} or Ab528 to the EGF receptor. The immunocomplexes were separated by SDS-polyacrylamide gels (5% acrylamide) and followed by Western blotting or *in vitro* kinase assays.

Western Blotting—Washed immunoprecipitates were mixed with SDS-gel sample buffer, heated at 95% for 5 min, and subjected to electrophoresis on 7.5% SDS-polyacrylamide gel. The gel-separated proteins were electrophoretically transferred onto nitrocellulose filters. Filters were first saturated for 1 h at 22 °C with blocking solution (10% low fat milk, 5% fetal calf serum in 20 mM Tris-HCl (pH 7.6) and 17 mM NaCl). Antisera were then added in the same solution, and incubation was carried out for 1 h. For detection the filters were washed three times (5 min each wash) with wash 1 (10% low fat milk, 0.05% Tween 20, 20 mM Tris-HCl (pH 7.6), and 17 mM NaCl) and reacted for 45 min at room temperature with horseradish peroxidase-conjugated protein A. The enzyme was removed by washing (as above) with wash 2 (0.05% Tween 20, 20 mM Tris-HCl (pH 7.6), and 17 mM NaCl). The filters were reacted for 1 min with a chemiluminescence reagent (ECL, Amersham) and exposed to an autoradiography film for 0.5–5 min.

In Vitro Autophosphorylation Assay—For autophosphorylation, 10 μl of HNTG containing 15 mM $MnCl_2$ and 5 μCi of [γ-^{32}P]ATP were added to the immunoprecipitate (final volume, 50 μl) and incubated for 15 min at 22 °C. The reaction was stopped by washing the immunoprecipitates with the high, medium, and low salt buffers. Then 20 μl of gel sample buffer was added, and the samples were boiled for 5 min. Proteins were separated by electrophoresis on SDS-polyacrylamide gels.

Binding of Radiolabeled EGF—Cells were plated at a density of 100,000 cells/well in 24-well dishes precoated with 10 μg/ml of human plasma fibronectin (Boehringer Mannheim). Cells were allowed to grow for 24 h to confluence. Murine EGF was iodinated by using the chloramine-T method to a specific activity of 100,000–200,000 cpm/ng. Confluent cells were washed with Dulbecco's modified Eagle's medium containing 25 mM HEPES (pH 7.4) and 0.1% of bovine serum albumin and then incubated with ^{125}I-EGF in the same buffer. Nonspecific binding was determined by the addition of 100-fold excess of native EGF together with ^{125}I-EGF to the binding experiment. After incubation for 2 h at 4 °C, the cells were placed on ice and washed three times with ice-cold PBS containing 0.1% bovine serum albumin. Labeled cells were lysed in 1 ml of 0.1 M NaOH, 0.1% SDS for 15 min at 37 °C, and the radioactivity was measured in a γ-counter to determine the amount of ligand bound to the cell surface.

Analysis of Ligand Association—NEC or TEC cells were incubated with the indicated concentrations of radiolabeled EGF in 0.25 ml of binding buffer for 1 min to 2 h on ice. The cells were rapidly washed twice with 1 ml of ice-cold binding buffer, dissolved in 0.1 M NaOH, 0.1% SDS, and the radioactivity was determined. Triplicate determinations were performed for each variable (time and ligand concentration). Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled EGF and did not exceed 10%. The association data were analyzed by determination of the amount of ligand bound at full saturation (B_∞) for each ligand concentration. The ratio of ligand bound at time t (B_t) to B_∞ was then calculated and described as a function of time. Alternatively, based on published theories of ligand association (Boeynaems and Dumont, 1980), the term $-\ln(1 - B_t/B_\infty)$ was calculated and represented as a function of time. If the association proceeds according to a simple one-site model a linear graph is expected in which the slope gives an indication of the kinetics parameters. Therefore, where possible (with TEC cells), the slopes of each straight line, resulting from association experiments performed with individual concentrations of ^{125}I-EGF, were described as the function of ligand concentration (see Fig. 9C). The resulting straight line corresponds to the following equation (Boeynaems and Dumont, 1980)

$$-\ln(1 - B_t/B_\infty) = (k_{on} \times [L] + k_{off}) \times t$$

where [L] is the concentration of the ligand. Accordingly, the values of the ligand association rate (k_{on}) and the ligand dissociation constant (k_{off}) can be calculated from the slope and the intercept, respectively, of the linear graph. When $-\ln(1 - B_t/B_\infty)$ is a nonlinear function with time (NEC cells, Fig. 8*B*), derivation of the kinetic constants cannot be performed directly according to the above described analysis. We therefore assumed the existence of two binding sites and used regression analysis to obtain the values of the association constants of site 1 (k_{on1}) and site 2 (k_{on2}), according to the following equation (Clark, 1933; Boeynaems and Dumont, 1980; Berkers *et al.*, 1991)

$$B_t = [R_1][L]/(K_d + [L]) \times (1 - e^{-(k_{on1} \times [L] + k_{off1})}) \times t + [R_2][L]/(K_d + [L]) \times (1 - e^{-(k_{on2} \times [L] + k_{off2})}) \times t$$

where B_t is the concentration of ligand bound at time t, [L] is ligand concentration, K_d is the equilibrium dissociation constant, [R_1] and [R_2] are the concentrations of receptors of each class, and k_{off1} and k_{off2} are the respective dissociation constants. The nonlinear regression procedure (NLIN) of SAS Institute, Inc. has been used.

Analysis of Ligand Dissociation—NEC and TEC cells were allowed to bind ^{125}I-EGF (30 ng/ml) for 2 h on ice. After a brief wash with binding buffer the cells were incubated for various periods of time with 1 ml of binding buffer with or without 500 ng/ml unlabeled EGF. The amount of cell-bound ligand at the end of the dissociation period was determined after a single wash with PBS. Triplicate determinations were performed, and the nonspecific binding was separately quantitated for each time point. Analysis of the dissociation data was performed according to the two-site receptor theory, which describes the amount of ligand bound at time t (B_t) by

$$B_t = B_{01} \times e^{-k_{off1}} \times t + B_{02} \times e^{-k_{off2}} \times t$$

where B_{01} and B_{02} are the amounts of ligand bound to receptor sites 1 and 2, respectively, before starting the dissociation phase, and k_{off1} and k_{off2} are the dissociation rates from site 1 and site 2, respectively. Based on this equation, expression of $\ln(B_t/B_o)$ *versus* time will result, in the case of a single site model, in a straight line whose slope corresponds to k_{off}. Similarly, a curvilinear graph that can be fitted to two straight lines is expected when analyzing dissociation from two distinct receptor classes. The experimental data obtained with TEC cells best fitted a one-site model, whereas the NEC cells yielded best fit with a two-site model, and were therefore accordingly analyzed.

Ligand Internalization Assay—Confluent monolayers of cells in 24-well dishes were preincubated for 30 min at 37 °C with 0.1 mM chloroquine. The cells were then washed and further incubated at 4 °C with 50 ng/ml ^{125}I-EGF in binding buffer containing chloroquine (0.1 mM). Receptor saturation was achieved after 2 h, and the cells were transferred to 37 °C for a variable length incubation that allowed internalization. At each time frame the cells were washed with binding buffer, and the surface-associated ligand was released by 7 min of incubation on ice with 0.15 M acetic acid containing 0.15 M NaCl (pH 2.7) (Yarden *et al.*, 1981). The residual cell-bound radioactivity was collected in 0.1 M NaOH, 0.1% SDS, and its radioactive content was determined. Nonspecific binding in the presence of 100-fold excess of unlabeled EGF was determined in parallel for each time point and respectively subtracted.

RESULTS

Functional Properties of the NEC (Val^{664}) and TEC (Glu^{664}) Hybrid Receptors—In the absence of a homogeneously purified ligand for the Neu receptor, we attempted to study the effect of the oncogenic mutation on the binding of a heterologous ligand. To this end we constructed chimeric proteins that include the extracellular domain of the EGF receptor and the transmembrane and cytoplasmic domains of Neu.

The exact point of fusion between the two proteins enabled us to conserve the overall protein structure of the extracellular domain of the EGF receptor and also allowed the use of the transmembrane domain of Neu (Fig. 1). The latter contained either valine at the position equivalent to residue 664 of the rat Neu or a glutamic acid residue. The expected two chimeric proteins were otherwise identical. When tested on a monolayer of normal Rat-1 fibroblasts by transfection, the mutant hybrid receptor, unlike the normal chimera, generated multiple foci indicative for retention of the transforming potential (Fig. 2*A*). However, in comparison with this function of the transforming rat *neu* oncogene, the foci that were induced by the latter gene appeared earlier (10 days after transfection, as compared with 2 weeks when using the chimera) and were also larger in size (Fig. 2*A*).

The plasmid vectors encoding each of the chimeric receptors were then introduced into murine fibroblasts that express no detectable receptor for EGF (Lev *et al.*, 1990). Single cell clones that stably express either the normal chimera (termed NEC for *N*eu-*E*GF receptor *c*himera) or the mutated protein (TEC, *t*ransforming *E*GF receptor *c*himera) were selected for high expression by binding assay of the radiolabeled ligand. When the transfected cells were tested for anchorage-independent growth in soft agar we found that the NEC cells gave rise to colonies of cells only in the presence of the ligand, whereas the TEC cells grew well in the semisolid medium even without the heterologous ligand (Fig. 2*B*). Fig. 3 depicts the results of immunoprecipitation analysis of the NEC and TEC proteins in the transfected cells. The TEC chimera displayed a doublet protein band, whereas NEC cells exhibited a single 185-kDa protein band that had a slightly retarded electrophoretic mobility. We attribute these differences to the higher rate of degradation of the mutation-containing protein (see below).

It has been shown previously that the transforming Neu protein is characterized by constitutive tyrosine kinase activity both in living cells (Yarden, 1990) and *in vitro* (Bargmann and Weinberg, 1988a). This is probably caused by permanent dimerization of the receptor (Weiner *et al.*, 1989). It was therefore important to determine whether these functional

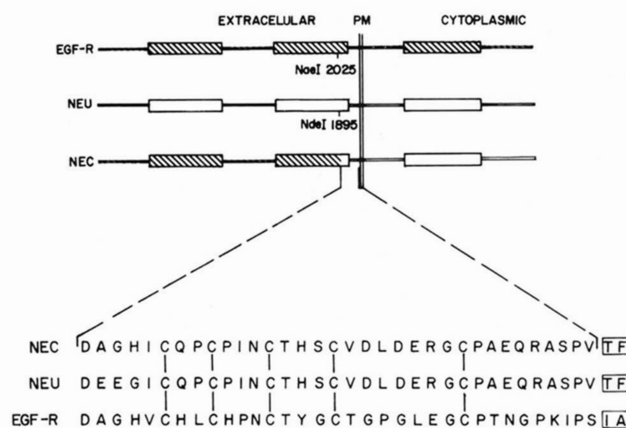

FIG. 1. **Schematic representation of the structures of the Neu-EGF receptor chimeric proteins.** The structures of the human EGF receptor (*EGF-R*) and the rat Neu protein are shown schematically. The *vertical double line* represents the plasma membrane (*PM*). Only the NEC chimeric protein is shown, but the TEC protein is identical except for a single point mutation at the transmembrane domain. *Boxes* at the extracellular domains indicate the cysteine-rich regions, whereas the cytoplasmic *boxes* represent the tyrosine kinase sequences. Amino acid sequences at the junction region are shown below the diagram with the preserved cysteine configuration demarcated with *vertical lines* and the amino termini of the transmembrane domains enclosed in *open boxes.*

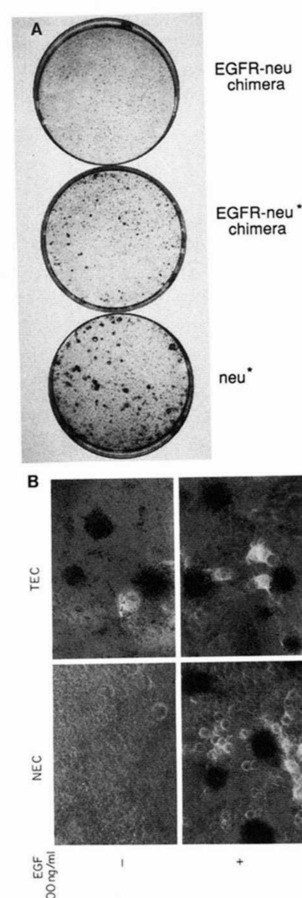

FIG. 2. **Transformation assays of the chimeric Neu-EGF receptor (*EGFR*) proteins.** *Panel A*, foci formation assay. Rat-1 cells in 90-mm plates were transfected with 10 μg of either the normal EGF receptor-*neu* plasmid or the mutant hybrid. For comparison a third plate was transfected with the transforming rat *neu* cDNA. After 3 weeks in culture the cells were fixed and stained by Giemsa staining. Shown are photographs of the stained plates. The results of a representative experiment, out of three, are shown. *Panel B*, soft agar colony formation assay. Murine fibroblasts that stably express the NEC or the TEC protein were cultured in agar-containing plates as described under "Experimental Procedures." EGF (100 ng/ml) was added to the indicated plates. The photomicrographs were taken after 3 weeks in culture. The black foci are large cell colonies that grew within the semisolid medium.

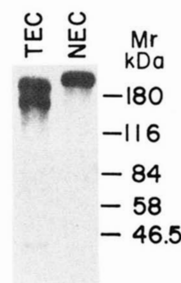

FIG. 3. **Expression levels of the NEC and TEC proteins.** The NEC and TEC clones of transfected murine fibroblasts were selected for overexpression of the NEC and TEC chimeric receptors, respectively. Confluent monolayers of cells growing in 30-mm plates were biosynthetically labeled with [^{35}S]methionine for 16 h at 37 °C. The chimeric proteins were then immunoprecipitated by using a monoclonal antibody directed to the extracellular domain of the EGF receptor (Ab528). An autoradiogram (15-h exposure) of the gel-separated immunocomplexes is shown.

consequences of the oncogenic mutation were preserved in the context of our chimeric molecule. Fig. 4 depicts the results of a tyrosine phosphorylation assay of the chimeric receptors in living cells in comparison with the intact receptor. Western blotting with antibodies to phosphotyrosine revealed that the NEC protein contained virtually no phosphotyrosine, whereas the TEC receptor was phosphorylated on tyrosine residues in living cells. Upon short incubation with EGF the NEC protein underwent extensive tyrosine phosphorylation, whereas the phosphotyrosine content of TEC remained unchanged. Remarkably, the extent of ligand-induced phosphorylation of the NEC protein was much higher than the phosphorylation exhibited by the TEC chimera, and it was similar to the level of phosphorylation seen with the intact EGF receptor. As shown in Fig. 4, the transforming Neu protein underwent limited but constitutive tyrosine phosphorylation.

We next compared the dimerization of the NEC and TEC proteins with that function of the rat Neu protein, with or without the oncogenic point mutation. A chemical cross-linking reagent was used to stabilize the dimeric receptor that was visualized either by an *in vitro* phosphorylation reaction or by Western blotting. The results of this analysis are given in Fig. 5. Evidently the transforming $p185^{neu}$ (Glu^{664}, B104-1-1 cells), unlike the normal protein (Val^{664}, G8 cells), displayed in Western blots a high molecular weight species which corresponded in size to a dimer form (Fig. 5*A*). This was not caused by the transformed phenotype of B104-1-1 cells as transformation of G8 cells by the *ras* oncogene did not result in dimerization of the normal Neu protein (Fig. 5*B*). In analogy with cells that overexpress the full-length normal Neu, unstimulated NEC cells exhibited a monomeric p185 molecule but no 360-kDa phosphorylated band corresponding to a receptor dimer (Fig. 5*C*). However, upon ligand stimulation a dimer protein band appeared, and it was recognized by antibodies to both major domains of the receptor. In contrast to the NEC protein, the transforming chimera displayed a dimeric receptor state with no dependence on the ligand (Fig. 5*C*). No dimeric receptor form was seen, in either NEC or TEC cells, in the absence of the cross-linking reagent (data not shown).

In summary of the functional analyses, the transforming TEC chimera formed dimers with no dependence on the ligand and also showed constitutive phosphorylation on tyrosine residues. Its normal counterpart, however, did not transform

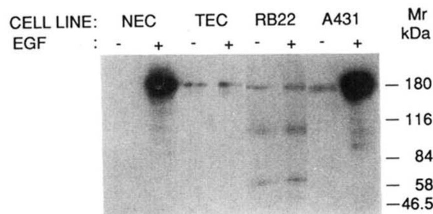

FIG. 4. **Tyrosine phosphorylation of the NEC and TEC proteins in living cells.** Subconfluent monolayers of cells in 30-mm dishes were incubated for 10 min at 22 °C with or without EGF (100 ng/ml). Cell lysates were then prepared and subjected to immunoprecipitation with either a monoclonal antibody specific to the human EGF receptor (Ab528; cell lines NEC, TEC, and A431) or the NCT rabbit antiserum directed to the carboxyl-terminal peptide of Neu (cell line RB22). The immunoprecipitates were extensively washed and separated by gel electrophoresis followed by Western blotting with an affinity-purified antibody to phosphotyrosine. The blots were detected with a chemiluminescence-based detection method (ECL, Amersham). The resulting autoradiogram (2-min exposure), is shown and the locations of molecular weight protein markers are indicated. The cell lines used, in addition to NEC and TEC, are the RB22 cells that overexpress the transforming Neu protein and the A431 human epidermoid carcinoma cells which overexpress the EGF receptor.

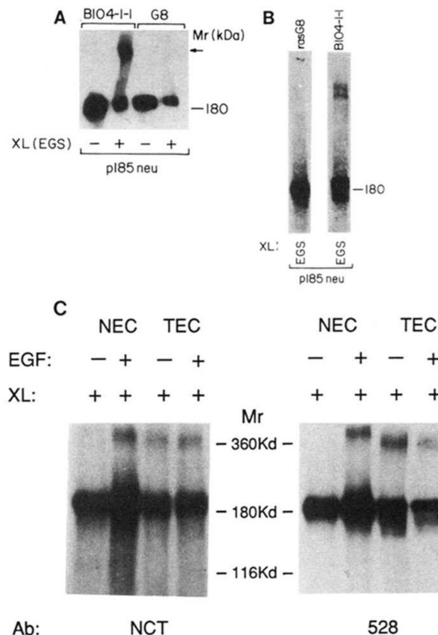

FIG. 5. **Covalent cross-linking of Neu proteins.** *Panel A*, murine fibroblasts that overexpress the transforming (B104-1-1 cells) or the normal $p185^{neu}$ (G8 cells) were grown to confluence on fibronectin-coated 150-mm plates. The monolayers were washed with PBS and incubated with PBS in the presence of dimethyl sulfoxide (1% final concentration) that contained or did not contain a cross-linking (*XL*) reagent (0.1 mM bis(succinimidyl succinate; *EGS*). The cells were incubated for 1 h at 22 °C and then subjected to immunoprecipitation of the Neu protein with the NCT antiserum. Immunocomplexes were resolved by electrophoresis in 5% acrylamide gel. Proteins were then electrophoretically transferred onto nitrocellulose and blotted with the NCT antiserum (1:500 dilution) followed by ^{125}I-protein A (400,000 cpm/ml). The nitrocellulose filter was exposed to film for 48 h. *Panel B*, confluent monolayers of B104-1-1 cells or *ras*-transformed G8 cells (*ras*-G8) were incubated with 0.1 mM bis(succinimidyl succinate) for 1 h at 22 °C, and cell lysates were then prepared. The normal (*ras*-G8 cells) or the transforming (B104-1-1 cells) $p185^{neu}$ was immunoprecipitated with specific antibodies (NCT), and the resultant immunocomplexes were subjected to an *in vitro* kinase assay. An autoradiogram of the gel-separated proteins was exposed for 12 h. *Panel C*, monolayers of NEC and TEC cells were preincubated with or without EGF (100 ng/ml) for 1 h at 4 °C, and a chemical cross-linker (EDAC, 15 mM) was subsequently added and incubated with the cells for 1 h at 22 °C. Cell lysates were immunoprecipitated with either an antibody to the Neu carboxyl terminus (NCT) or a monoclonal antibody to the EGF receptor (Ab528) as indicated. The extensively washed immunoprecipitates were subjected to an *in vitro* kinase assay and separated by SDS-polyacrylamide gel electrophoresis. The resulting autoradiogram (12-h exposure) is shown along with the locations of molecular weight marker proteins.

cells, exhibited no dimer form and no phosphorylation on tyrosine residues unless stimulated by the heterologous ligand.

Ligand Binding Analyses: The Oncogenic Receptor Displays Higher Affinity—To study the effect of the single amino acid change on the interaction with the ligand we performed comparative equilibrium binding assays with radiolabeled EGF. The results of this experiment are given in Fig. 6 as saturation curves and their respective Scatchard plots. Evidently NEC cells expressed 2.2×10^5 binding sites/cell whereas TEC cells expressed 3×10^5 sites/cell (calculated from the intercepts with the horizontal axis of the Scatchard plots). More important, the half-saturating concentrations of the ligand were significantly different: 11.2 and 0.9 nM for NEC and TEC cells, respectively. Scatchard analysis revealed also a qualitative difference; whereas the NEC protein exhibited a curvilinear plot, a straight line was observed with the TEC cells. A likely interpretation is therefore that the NEC

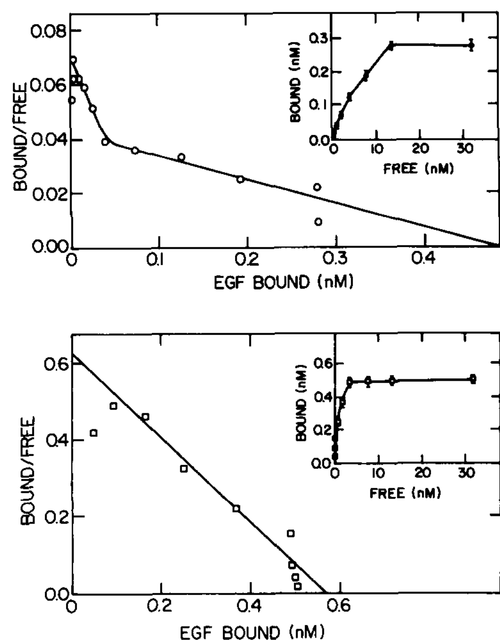

FIG. 6. Scatchard analysis of ^{125}I-EGF binding to intact cells expressing the chimeric Neu-EGF receptor proteins. Confluent monolayers of NEC (*upper panel*) or TEC cells (*lower panel*) in 24-well dishes were incubated for 2 h at 4 °C with different concentrations of ^{125}I-EGF. Binding results were analyzed by the Scatchard method and also by saturation curves (*insets*). Triplicate determinations of total binding were performed, and the nonspecific binding in the presence of 100-fold excess of unlabeled ligand was determined for each concentration and respectively subtracted. *Bars* represent the standard errors. Similar results were obtained in three additional experiments.

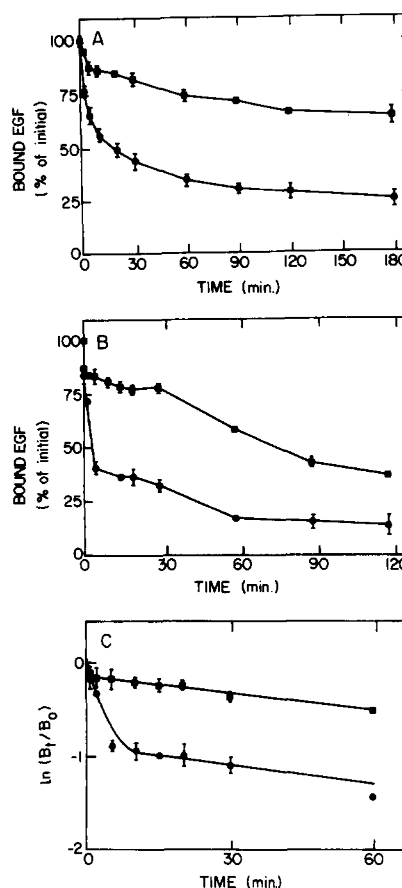

FIG. 7. Kinetics of ligand dissociation from NEC and TEC cells. Confluent monolayers of NEC (*circles*) or TEC cells (*squares*) in 24-well dishes were incubated with 30 ng/ml ^{125}I-EGF for 2 h at 4 °C. The monolayers were then washed, and 1 ml of binding buffer was subsequently added. The buffer either contained no unlabeled EGF (*panel A*) or 500 ng/ml EGF (*panel B*). Dissociation was monitored for 3 h at 4 °C. The data represented in *panel B* were analyzed as described under "Experimental Procedures" by plotting ln (B_t/B_o) *versus* time, where B_t is the concentration of ligand bound at time t and B_o is the concentration of ligand bound before starting the dissociation. The results are shown as a semilogarithmic plot (*panel C*). Each point represents the mean ± S.E. (n = 3). The experiment was repeated three times with essentially the same results.

protein, like the wild-type EGF receptor, exists in two populations of binding sites, a minor high affinity class (~22,000 sites/cell, K_d = 0.7 nM), and a major population of relatively low affinity receptors (~200,000 sites/cell, K_d = 11.2 nM) ligand affinities were calculated from the slopes of the Scatchard plots. On the other hand, all of the mutant chimeric receptors of TEC cells belonged to a homogeneous population of binding sites with an affinity similar to the minor population of the NEC receptors.

The affinity constants obtained under equilibrium conditions reflect both the k_{on} and the k_{off} of the ligand from its binding site. We therefore sought to determine if the mutation-induced difference in ligand binding affinity was mostly a result of one of these rate constants or was a mixed effect. The dissociation of the ligand from NEC and TEC cells was followed at 4 °C after the cells were labeled with 30 ng/ml ^{125}I-EGF for 2 h on ice, to avoid internalization. Following a short wash, dissociation was initiated by adding fresh binding buffer that contained 500 ng/ml unlabeled ligand or no ligand. In both cases marked differences were observed between the dissociation curves of NEC and TEC cells, but in the presence of unlabeled EGF the rates exhibited by both cell lines were significantly higher (Fig. 7). To avoid an effect of ligand reassociation, we based our calculations on dissociation experiments that were performed in the presence of unlabeled EGF (Fig. 7*B*). The data obtained were analyzed by plotting the natural logarithm of the fractional receptor occupancy, B_t/B_o, as a function of time, where B_t is the amount of ligand bound at time t and B_o is the amount of ligand bound before starting dissociation (Fig. 7*C*). The negative value of the slope of such plots should indicate the dissociation constant. Indeed, a straight line corresponding to a k_{off} value of 2×10^{-4} s^{-1} characterized dissociation from the mutant receptor. The wild-type protein, however, displayed a biphasic curve. We therefore assumed the existence of two receptor sites and analyzed the data according to the two-site theory (Boeynaems and Dumont, 1980). Following this model, we found a fast dissociating site with a k_{off} value of 2×10^{-3} s^{-1}, and a 10-fold slower dissociating component with a rate constant of 2×10^{-4} s^{-1} (Fig. 7*C*). A plausible interpretation of these data would then attribute the high rate of ligand dissociation to the low affinity class of the wild-type receptors (Fig. 6), whereas the high affinity receptors, either mutants (TEC) or the minor class of the wild-type proteins, appear to be characterized by significantly lower rates of ligand dissociation.

The kinetics of ligand association with the NEC and TEC proteins were determined at 4 °C to avoid differences caused by the higher rate of internalization of the mutant receptor (see below). Surprisingly, at all concentrations of EGF used in the binding assays (in the range of 0.5–8 nM), we observed faster receptor saturation with the NEC protein as compared with the mutant chimera. This is exemplified in Fig. 8, which depicts the results of an experiment performed with 15 ng/ml ^{125}I-EGF. The maximal binding capacity (B_∞) at this concentration of EGF was calculated from a long term saturation

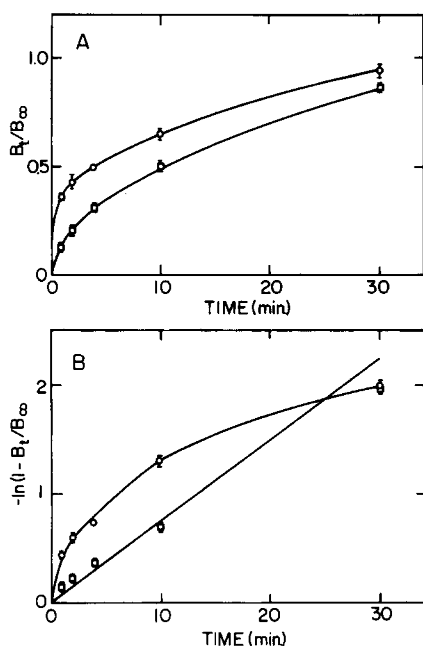

FIG. 8. **Association kinetics of EGF binding to NEC and TEC cells.** Monolayers of NEC (*circles*) or TEC cells (*squares*) in 24-well dishes were allowed to bind ^{125}I-EGF at a concentration of 15 ng/ml. Bound ^{125}I-EGF was determined at various time points, the nonspecifically bound fraction subtracted, and the results calculated as fractional receptor occupancy, B_t/B_∞, where B_t is the concentration of ligand bound at time t and B_∞ is the maximal concentration of ligand bound at complete saturation. The data presented in *panel A* are shown in *panel B* as a semilogarithmic plot in which the negative values of ln $(1 - B_t/B_\infty)$ were plotted *versus* time. *Bars* represent the standard errors for triplicate determinations.

experiment, and the ratio of ligand bound at time t (B_t) and B_∞ was then calculated and plotted as a function of time. As shown, NEC cells exhibited higher rate of ligand association at 4 °C. Association analysis can also provide an indication as to the existence of one receptor class as opposed to two or more distinct sites. By using theories of association kinetics analysis described by Clark and others (Clark, 1933; Boeynaems and Dumont, 1980) presentation of association data as $-\ln(1 - B_t/B_\infty)$ *versus* time should yield a linear relationship in the case of a homogeneous receptor population, whereas a curvilinear plot is indicative of a two-, or more, site model. Transformation of the data given in *panel A* of Fig. 8 into such semilogarithmic plots revealed a straight line in the case of TEC and a curvilinear graph in the case of NEC (Fig. 8*B*). We therefore concluded that ligand binding to the transforming chimera of TEC cells proceeds according to a one-site model, whereas the valine version of the Neu-EGF receptor chimera exists in two (or more) receptor classes. This conclusion is consistent with the Scatchard analysis of the chimeric proteins (Fig. 6).

Kinetics parameters can be determined experimentally in the case of a one-site model, unlike the two-site model which requires the use of linear regression and curve fitting. Such an analysis, using the nonlinear regression procedure of SAS (see "Experimental Procedures") and the data of Fig. 8*B* yielded a single association rate in the case of TEC (k_{on} 0.6 × 10^6 M^{-1} · s^{-1}) and two very similar constants for NEC: 1.4 × 10^6 and 1.2 × 10^6 M^{-1} · s^{-1} which presumably correspond to the low and the high affinity states, respectively. The latter values are given in parentheses in Table I to indicate their derivation from mathematical manipulations. To determine more precisely the kinetic parameters of the mutated receptor, the association experiment was repeated with different EGF concentrations and analyzed as in Fig. 8. The results of this experiment are shown in Fig. 9. By plotting the slopes of the lines obtained in panel *B* of Fig. 9 as a function of ligand concentration, k_{off} could be determined from the intercept with the *y* axis, and the slope of the graph indicated k_{on}. Accordingly, this analysis (Fig. 9*C*) yielded a dissociation constant of 0.25 × 10^{-3} s^{-1} and an association rate of 0.6 × 10^6 M^{-1} · s^{-1} for the TEC protein. The value of k_{off} that was determined directly in dissociation experiments (0.21 × 10^{-3} s^{-1}; Fig. 7*C*) is in agreement with the result obtained in the association experiment. The apparent K_d determined by the ratio of the kinetically measured rate constants (k_{off}/k_{on}) was calculated as 0.35–0.4 nM, which is in agreement with the 0.9 nM value obtained in the Scatchard analysis. Less satisfactory were the calculated kinetic K_d values for the NEC protein. These were smaller than the equilibrium K_d, probably reflecting the inaccurate derivation of the k_{on} values (Fig. 8*B*). A summary of the binding parameters of the Neu-EGF receptor chimeric proteins is given in Table I and compared with the values reported for the wild-type EGF receptor.

Receptor Degradation and Ligand Endocytosis—The analysis of ligand-receptor interactions and the effect of the transmembrane mutation were extended to cellular routing of ligand-receptor complexes. Examination of receptor degradation by pulse-chase biosynthetic labeling with [^{35}S]methionine revealed that the mutant chimeric receptor underwent faster turnover relative to the valine variant (Fig. 10). Thus, whereas the NEC receptor displayed a half-life of 6.5 h, the transforming chimera exhibited twice as rapid a rate of endocytosis and degradation. Binding of the ligand, however, accelerated the rate of turnover of the nontransforming chimera up to the rate of the mutant protein. Nevertheless, the ligand had no effect on the rate of degradation of the mutant receptor (Fig. 10), perhaps indicating that the latter underwent endocytosis at the maximal rate.

The rates of endocytosis of EGF by the mutant and the wild-type receptors were compared by removal of the surface-bound EGF under acidic conditions (Yarden *et al.*, 1981). In initial experiments we noticed that such analysis was complicated by apparently different rates of ligand degradation, exocytosis, and dissociation from the two cell lines. Therefore, to analyze ligand endocytosis separately we inhibited intracellular degradation with the lysosomotropic drug chloroquine and used continuous incubation with ^{125}I-EGF. Under these conditions both NEC and TEC cells gradually accumulated the ligand intracellularly at practically identical rates (Fig. 11), consistent with their similar rates of endocytosis and degradation in the presence of the ligand (Fig. 10).

DISCUSSION

The present study addressed the functional consequences of the oncogenic mutation in the Neu receptor on its interactions with a ligand. Since the point mutation affects the oligomerization state of p185^{neu} (Fig. 5; Weiner *et al.*, 1989), we hoped that by approaching this question we will gain insights into the more general, but still open, issue of the relationships between receptor oligomerization and heterogeneity of ligand binding affinities. Although all the growth factor receptors with associated tyrosine kinase activity undergo ligand-induced dimerization (reviewed by Ullrich and Schlessinger, 1990), causal relationships between receptor dimerization and the interconversion of high and low affinity ligand binding sites, in living cells, have not been examined directly.

As expected, ligand binding to the unmutated Neu-EGF receptor chimera displayed binding characteristics that were

TABLE I

Summary of binding parameters of the NEC and TEC proteins in comparison with the EGF receptor

Listed are the parameters obtained from the data presented in Fig. 6 (K_d, equilibrium), Fig. 7C (k_{off}), and Figs. 8 and 9 (k_{on} values). The values in parentheses were derived from a nonlinear regression analysis (Fig. 8B). The k_{off} values of TEC were obtained either directly from dissociation analysis (Fig. 7C) or calculated from association results (Fig. 9). The apparent K_d values (kinetics) were calculated as the dividend of the dissociation and association rates. Receptor numbers were derived from the intercept points of the respective Scatchard plots (Fig. 6). Each value was determined in at least three separate experiments in which triplicates were used for every time or concentration point. For comparison, reported binding parameters of the EGF receptor are also enlisted. These were taken from Bellot *et al.* (1990) (values labeled with [a]) or Berkers *et al.* (1991) (values labeled with [b]).

	K_d equilibrium	k_{off}	k_{on}	K_d kinetics	No. of receptor sites
	nM	$(s^{-1}) \times 10^{-3}$	$(M^{-1} \times s^{-1}) \times 10^6$	*nM*	%
NEC					
High	0.7	0.2	(1.2)	(0.15)	10
Low	11.2	2.0	(1.4)	(1.5)	90
TEC	0.9	0.21–0.25	0.6	0.35–0.4	100
EGF-R					
High	0.17[a]	2.9[a]	9.8[a]	0.3[a]	1.8[a]
	0.12[b]	0.35[b]	0.6[b]	0.6[b]	47[b]
Low	14[a]	2.9[a]	0.25[a]	11.4[a]	98.2[a]
Fast	7.1[b]	8.1[b]	3.3[b]	2.4[b]	24[b]
Slow	7.1[a]	0.16[b]	0.03[b]	4.9[b]	29[b]

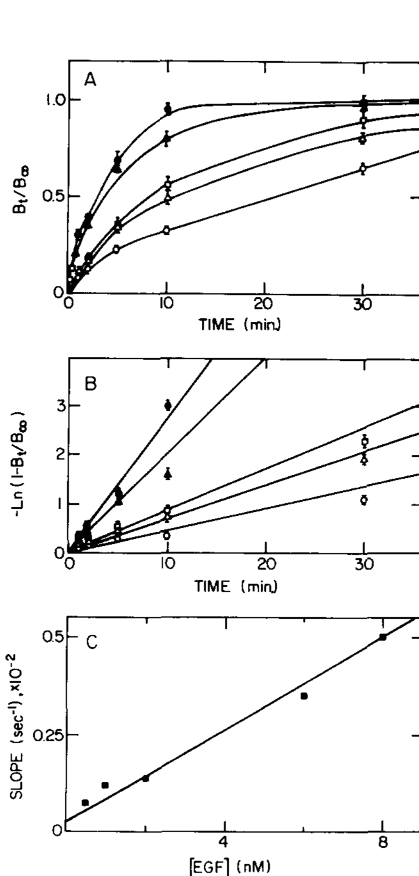

FIG. 9. **Association kinetics of the binding of various concentrations of EGF to TEC cells.** Confluent monolayers of TEC cells in 24-well dishes were incubated with ^{125}I-EGF for the indicated periods of time. EGF was used at final concentrations of 0.5 ng/ml (*open circles*), 1 ng/ml (*open triangles*), 2 ng/ml (*squares*), 6 ng/ml (*closed triangles*), and 8 ng/ml (*closed circles*). Maximal ligand binding (B_∞) was separately determined for each concentration of EGF. *Panel A* shows the dividend of ligand bound at time *t* (B_t) and B_∞ as a function of time. The same data are presented as $-\ln(1 - B_t/B_\infty)$ in *panel B*. The slopes of the plots obtained in *panel B* are depicted as a function of ligand concentration in *panel C*. Each point represents the mean of a triplicate ± S.E. The experiment was repeated three times.

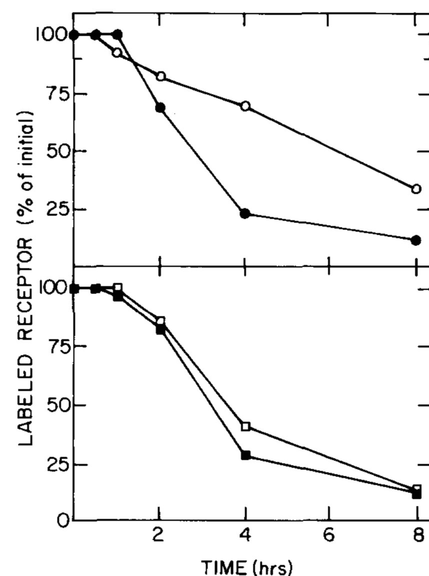

FIG. 10. **Degradation rates of NEC and TEC proteins.** Subconfluent monolayers of NEC (*circles, upper panel*) and TEC cells (*squares, lower panel*) grown in 30-mm dishes were biosynthetically labeled with [^{35}S]methionine (50 μCi/ml) for 16 h at 37 °C. The monolayers were then washed and chased with fresh medium that either contained EGF (100 ng/ml, *closed symbols*) or no addition (*open symbols*). After the indicated incubation periods at 37 °C the Neu-EGF receptor chimeric proteins were immunoprecipitated by using Ab528 to the EGF receptor. The level of each receptor was determined by densitometry of autoradiograms of the gel-separated immunocomplexes and expressed as a fraction of the initially labeled respective receptor.

at least qualitatively similar to those exhibited by the wild-type EGF receptor. Thus, like the EGF receptor (Defize *et al.*, 1989; Berkers *et al.*, 1991, Bellot *et al.*, 1990; Wiley, 1988, reviewed by Schlessinger, 1986), the NEC protein displayed a curvilinear Scatchard plot (Fig. 6), a biphasic ligand dissociation curve (Fig. 7), and a calculated nonlinear kinetic of ligand association (Fig. 8). These characteristics are all consistent with the existence of heterogeneous affinity states that can be interpreted in terms of two kinetically distinct populations of receptors. Quantitatively, NEC differs from the EGF receptor in the ratio between the numbers of the high and low affinity sites (Table I). Other differences relate to the values

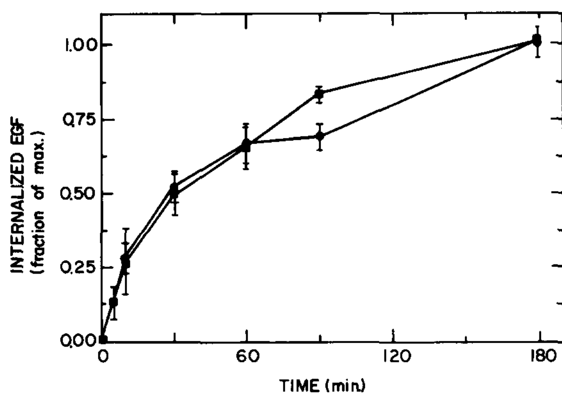

FIG. 11. **Internalization of ^{125}I-EGF into NEC and TEC cells.** Monolayers of NEC (*circles*) or TEC cells (*squares*) in 24-well dishes were preincubated for 30 min with 0.1 mM chloroquine and then saturated with ^{125}I-EGF at 4 °C. The rates of ligand internalization at 37 °C were determined as described under "Experimental Procedures." Duplicate determinations were performed. Their averages and ranges (*bars*) are expressed as fractions of the cell-associated radioactivity after 3 h of incubation. The experiment was repeated three times.

of equilibrium and kinetic parameters, as detailed in Table I. Although this can be attributed to the fusion of the EGF binding domain with the Neu protein, the large variation among many reports on the binding parameters of the EGF receptor may also account for the observed differences. For example, two distinct low affinity EGF receptor sites were observed in HeLa cells (Berkers *et al.*, 1991), whereas others reported affinities that are practically identical to those of NEC (Defize *et al.*, 1989).

In contrast with the valine version of the chimeric receptor, the oncogenic glutamate version displayed homogeneous binding characteristics, as was reflected in linear Scatchard plot (Fig. 6), dissociation curve (Fig. 7), and association kinetics (Fig. 8). This behavior can be interpreted as indicating the existence of a single population of ligand binding sites with an affinity comparable to the minor high affinity population of NEC. Because of the homogeneous nature of ligand binding to TEC, kinetic parameters could be derived for it (Fig. 9), unlike NEC. Surprisingly, this analysis revealed that the single point mutation not only reduced the rate of ligand dissociation but also slightly inhibited the rate of ligand association (Fig. 8). One possible interpretation of the reduced rate of ligand association is that the oncogenic mutation imposed a steric effect which caged the binding cleft and thereby inhibited ligand entry as well as exit.

In light of the different characteristics of ligand binding to two receptors, which differ in only 1 out of about 1,250 amino acids, it is relevant to address the molecular mechanism. The data presented in this paper (Fig. 5) as well as in a report by Weiner *et al.* (1989) and two structural models of p185^{neu} (Sternberg and Gullick, 1990; Brandt-Rauf *et al.*, 1990) suggest the possibility that homogenization of the normally heterogeneous distribution of affinity states is caused by constitutive oligomerization of the mutant receptors. It then follows that the valine version of Neu-EGF receptor exists in both receptor monomers and receptor dimers corresponding to low and high affinity ligand binding sites, respectively. The ratio between these receptor states is 9:1 according to Scatchard analysis (Table I), which is in excellent agreement with the calculated distribution of the transmembrane domain between an α-helix configuration (dimer-forming) and a bent (monomer-forming) structure (Brandt-Rauf *et al.*, 1990). Based on these considerations, we assume that the normal Neu-EGF receptor chimera, and by analogy the valine version of the full-length p185^{neu}, exists in equilibrium between monomers and preexisting dimers. Ligand association, according to this model, proceeds more rapidly to the monomeric form. However, because of a lower rate of ligand dissociation from the dimeric form, the latter is overall kinetically preferred.

If an analogy can be drawn between the chimeric Neu proteins and the wild-type EGF receptor, it would then imply that the minor high affinity population represents catalytically active dimeric receptors. This simple model has been discussed previously by us (Yarden and Schlessinger, 1987b). However, it has been later challenged by others (Koland and Cerione, 1988; Northwood and Davis, 1988; but see also Verheijden *et al.*, 1991), probably because the interconversion between high and low affinity states also occurs via alternative mechanisms involving protein kinases (Walker and Burgess, 1991) and receptor autophosphorylation sites (Livneh *et al.*, 1986).

On the basis of the biochemical and oncogenic potentials of NEC and TEC, we propose that the high affinity dimeric receptor state is the biologically active species. Accordingly, the dimeric receptor, unlike the dispersed monomers, transmits tyrosine kinase signals that culminate in accelerated cell proliferation and eventually phenotypic transformation (Fig. 2). Consistent with this notion, the high affinity subpopulation of EGF receptor has been shown to comprise the biologically active class of receptors, on the basis of experiments with class-specific monoclonal antibodies (Defize *et al.*, 1989; Bellot *et al.*, 1990). In the case of Neu, not only a ligand and a specific mutation, but also overexpression at the cell surface may induce the formation of the biologically active dimeric state (Brandt-Rauf *et al.*, 1990). This third mechanism may have clinical implications since overexpression of Neu, which also occurs naturally in many human adenocarcinomas (reviewed in Slamon *et al.*, 1989 and Gullick, 1990), entails tumorigenesis in cellular and animal model systems (Hudziak *et al.*, 1987; DiFiore *et al.*, 1987). Moreover, Neu overexpression is biochemically coupled to high autokinase activity (Lonardo *et al.*, 1990; Peles *et al.*, 1991) and tyrosine phosphorylation of cytoplasmic proteins, including phospholipase Cγ (Peles *et al.*, 1991). We therefore speculate that this mechanism may allow elevated sensitivity of Neu-overexpressing clones of tumor cells to the endogenous ligand through a dimerization-mediated increase in binding affinity.

In conclusion, our results provide, for the first time, experimental evidence from living cells which suggests causal relationships between the formation of high affinity ligand binding and the process of receptor dimerization and also correlates these relationships with the oncogenic potential of Neu proteins.

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