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

IMPLICATIONS FOR THE GENERATION OF SITE HETEROGENEITY*

(Received for publication, February 18, 1992)

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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 p185neo, 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)

* This work was supported in part by National Institutes of Health Grant CA51712 and by a grant from The Wolfson Foundation, administered by the Israel Academy of Sciences and Humanities. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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encodes a 185-kDa transmembrane glycoprotein that is highly homologous to but yet distinct from the epidermal growth factor (EGF) 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 dimeric 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 p185neo for the interaction with its presumed ligand molecule are yet unknown. The existence of a ligand molecule of p185neo 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., 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 transduction studies.

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)ethylenediamine); Ab, antibody; NCT, Neu carboxyl terminus.

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transduction studies (Sistonen et al., 1988; 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 close 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, 1987; 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<sup>Neu</sup> autonomously creates receptor dimers (Weiner et al., 1988; Sternberg and Gallic, 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 Biosertor (Rehovot, Israel) and by Toyocho (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<sup>Neu</sup>, B104 (Bargmann et al., 1989; Peles et al., 1989; Sternberg and Gullick, 1990; Yarden et al., 1991) and some lymphokine receptors, were transfected by the calcium phosphate precipitation method with 10 pg of plasmid DNA/90-mm plate. After 18 h the cells were transfected by the calcium phosphate precipitation method with either pCMV-Neu 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 to the cells. The resulting 20% of both NEp and TEC chimeric genes were sequenced to ensure integrity of the open reading frames. In contrast with the receptor for nerve growth factor (Sutter et al., 1979) and some lymphokine receptors, ligand binding to homodimers of the EGF receptor 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<sup>Neu</sup> autonomously creates receptor dimers (Weiner et al., 1988; Sternberg and Gallic, 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.
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 the receptor turnover rates, the incubation with [35S]methionine was followed by a 10-min chase with fresh medium containing 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 quantification 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 and incubated with 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(55) or Ah528 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°C 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). Antibodies 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 h 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 MnCl2 and 5 μCi of [γ-32P]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. One million cells were washed twice 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 125I-EGF in the same buffer. Non specific binding was determined by the addition of 100-fold excess of native EGF together with 125I-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 immunoprecipitated 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). The 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 (Bmax) for each ligand concentration. The ratio of ligand bound at time t (Bt) to Bmax was then calculated and plotted as a function of time. Alternatively, based on the kinetic theories of ligand association (Boeynaems and Dumont, 1980), the term \[-\ln(1 - B_t/B_{max})\] 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 125I-EGF, were described by the equation of a straight line whose slope corresponds to the following equation (Boeynaems and Dumont, 1980):

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

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 \[-\ln(1 - B_t/B_{max})\] is a nonlinear function with time (NEC cells, Fig. 8B), 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):

\[B_t = [R_1][L]/(K_2 + [L]) \times (1 - e^{-k_{off1}[L] + k_{on1}) \times t\]

where \(B_t\) is the concentration of ligand bound at time t, [L] is ligand concentration, K_2 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 125I-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

\[B_t = B_{max} \times e^{-k_{off1}t} + B_{max} \times e^{-k_{off2}t}\]

where B_{max} and B_{off} 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_{off}] versus time will result in, in case of a single site model, in a straight line whose slope corresponds to k_{off}, since a linear graph that can be fitted to two straight lines is expected when analyzing dissociation from two different receptor classes. The experimental data obtained with TEC cells best fitted a one-site model, whereas the NEC cells yielded the 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 125I-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 mM 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^{105})** and** TEC (Glu^{106})** 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. 2A). 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. 2A).

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 Neu-EGF receptor chimera) or the mutated protein (TEC, transforming EGF receptor chimera) 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. 2B). 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

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**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 chimera 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.

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**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.

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**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 [35S]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 phosphotyrosine content of TEC remained unchanged. Remarkably, the extent of tyrosine 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 (Glu64, B104-1-1 cells), unlike the normal protein (Val64, G8 cells), displayed in Western blots a high molecular weight species which corresponded in size to a dimer form (Fig. 5A). 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. 5B). 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. 5C). 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. 5C). 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 antisera 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 antisera. Immunocomplexes were resolved by electrophoresis in 5% acrylamide gel. Proteins were then electrophoretically transferred onto nitrocellulose and blotted with the NCT antisera (1:500 dilution) followed by 125I-protein A (400,000 cpm/ml). The nitrocellulose filter was exposed to film for 1 h.

**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 × 10^9 binding sites/cell whereas TEC cells expressed 3 × 10^9 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 cells, exhibited no dimer form and no phosphorylation on tyrosine residues unless stimulated by the heterologous ligand.
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 $4^\circ$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. 7B). The data obtained were analyzed by plotting the natural logarithm of the fractional receptor occupancy, $B_t/B_0$, as a function of time, where $B_t$ is the amount of ligand bound at time $t$ and $B_0$ is the amount of ligand bound before starting dissociation (Fig. 7C). 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 $2 \times 10^{-4}$ 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 $2 \times 10^{-4}$ s$^{-1}$, and a 10-fold slower dissociating component with a rate constant of $2 \times 10^{-5}$ s$^{-1}$ (Fig. 7C). 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 $4^\circ$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 ($B_{max}$) at this concentration of EGF was calculated from a long term saturation

![Fig. 6. Scatchard analysis of $^{125}$I-EGF binding to intact cells expressing the chimeric Neu-EGF receptor proteins.](image)

![Fig. 7. Kinetics of ligand dissociation from NEC and TEC cells.](image)
experiment, and the ratio of ligand bound at time t ($B_t$) and $B_\infty$ 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. 8B). 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).

Kinetic 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. 8B 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. 9C) 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. 7C) is in agreement with the result obtained in the association experiment. The apparent $K_a$ determined by the ratio of the kinetically measured rate constants ($k_{on}/k_{off}$) 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 $K_a$ values for the NEC protein. These were smaller than the equilibrium $K_a$, probably reflecting the inaccurate derivation of the $k_{on}$ values (Fig. 8B). 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 [35S]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 [125I]-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 p185Neu (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
Ligand Binding to Neu

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 (Kd, equilibrium), Fig. 7C (koff), and Figs. 8 and 9 (kon values). The values in parentheses were derived from a nonlinear regression analysis (Fig. 8B). The koff values of TEC were obtained either directly from dissociation analysis (Fig. 7C) or calculated from association results (Fig. 9). The apparent Kd 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 *) or Berkers et al. (1991) (values labeled with †).

|       | Kd equilibrium | koff | kcon | Kd kinetics | No. of receptor sites |
|-------|----------------|------|------|-------------|-----------------------|
| NEC   | nM             | (s⁻¹) x 10⁻³ | (M⁻¹ x s⁻¹) x 10⁸ | nM          | %                     |
| 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*          | 2.9* | 9.8* | 0.3*        | 1.8*                  |
| Low   | 0.12†          | 0.35† | 0.6* | 0.6*        | 47*                   |
| Fast  | 7.1†           | 8.1* | 3.3* | 2.4*        | 24*                   |
| Slow  | 7.1†           | 0.16† | 0.05* | 4.9*        | 29*                   |

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 [³⁵S]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.

Fig. 10. Degradation rates of NEC and TEC proteins. Sub-confluent monolayers of NEC (circles, upper panel) and TEC cells (squares, lower panel) grown in 30-mm dishes were biosynthetically labeled with [³⁵S]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...
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

\[ p_{185} \]

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 \( \alpha \)-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

\[ p_{185} \]

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 (Lorando et al., 1990; Peles et al., 1991) and tyrosine phosphorylation of cytoplasmic proteins, including phospholipase C\( \gamma \) (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.

Acknowledgments—We thank Robert Weinberg, Mien-Chie Hung, and David Stern for cell lines, John Mendelsohn for Ab528, Sigitat Pias for technical help, and Miriam Fagan for typing the manuscript.

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