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Ligand and p185^{c-neu} density govern receptor interactions and tyrosine kinase activation

(receptor dimerization/interactions of p185^{c-neu} receptors/neu-activating factor)

ARABINDA SAMANTA*, CHARLES M. LEVEA, WILLIAM C. DOUGALL, XIAOLAN QIAN, AND MARK I. GREENE

Center for Receptor Biology, Department of Pathology and Laboratory Medicine, and the Johnson Foundation, University of Pennsylvania, Philadelphia, PA 19104

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ABSTRACT **The *neu* protooncogene (also known as c-*erbB2*, *NGL*, and *HER2*) encodes a 185-kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity that resembles the receptor for epidermal growth factor. The p185 gene and protein were originally identified in the brain and are thought to play a critical role in neurogenesis. Aberrant c-erbB2 protein overexpression also occurs in several human adenocarcinomas. A ligand for p185, neu-activating factor (NAF), specifically binds to neu receptor and increases the p185^{c-neu} tyrosine phosphorylation *in vitro* and *in vivo* in a dose-dependent manner. We now show that NAF specifically binds to purified p185 expressed in baculovirus. Direct binding analysis showed that NAF binds with high affinity (K_d = 1.3 nM). We have investigated changes in the structure and association state of baculovirus-produced neu holoreceptor that are induced by ligand binding. In this study, we used sucrose gradients to show that purified p185^{c-neu} exists mainly in the monomeric form at low concentrations, whereas at higher concentrations p185^{c-neu} exists as dimers or multimers. At low concentrations, but in the presence of ligand, p185^{c-neu} sediments as a dimeric or multimeric form. Monomer–oligomer interconversion is absolutely ligand dependent at low receptor concentrations. The high molecular weight form of the receptor is enzymatically more active, as a consequence of ligand-driven activation of the receptor kinase. Oncogenic p185^{neu} receptors sediment predominantly as high molecular weight forms and have constitutively active kinases.**

Polypeptide growth factors stimulate a cascade of events that culminates in cell growth and differentiation (1, 2). p185^{c-neu} is a developmentally regulated tyrosine kinase that plays an important role in the central nervous system (3). For the most part, the intracellular mitogenic signal-transduction pathway is incompletely understood. p185^{c-neu} may have unusually complex activation pathways since it can form homomeric and heteromeric associations (4). It is known, for example, that p185 can associate with the epidermal growth factor receptor and then be activated when epidermal growth factor binds to its receptor (4).

A variety of molecules have been implicated as growth factors for p185^{c-neu}. The recently reported neu-differentiation factor (NDF) is a 44-kDa glycoprotein secreted by ras-transformed Rat1-EJ cells and is now thought to be a ligand for an undefined structure that can associate with p185^{c-neu} (5–7). Indeed this may represent still another heteromer between p185 and a yet to be identified member of the erbB family of receptors. NDF induces cell polyploidy, inhibits cell growth, and arrests the cell cycle at the late S or G_2/M phases. Another molecule, heregulin (45 kDa), has been derived from the MDA-MB-231 human breast carcinoma cell line (8). Both NDF and heregulin have been cloned and sequenced and are homologous. Still another 30-kDa glycoprotein has been isolated from the medium of the MDA-MB-231 cell line and shows some sequence identity with heregulin (9). A factor of 25 kDa has been isolated from bovine kidney and is mitogenic for NIH 3T3, DHFR-G8, and A431 cells (10). We have isolated and characterized a neu-activating factor (NAF) from a transformed human T-cell line (11). The NAF ligand specifically interacts with the neu extracellular domain, resulting in neu-specific tyrosine kinase activation, receptor dimerization, internalization, and concomitant potentiation of the growth of neu receptor-expressing cell lines. NAF can be distinguished from those ligands described above since it can bind directly to soluble p185 and it has a distinct M_r (15,000–17,000; A.S. and M.I.G., unpublished results) and induces p185 down modulation from the cell surface.

Two different models exist for the activation of tyrosine kinase membrane-spanning receptors. Intramolecular models of activation suggest that binding of ligand to the extracellular domain induces a conformational change that is propagated through the transmembrane segment to produce the active conformation of the kinase (32). Another model proposes an intermolecular model of activation. In this model, binding of ligand shifts the equilibrium between inactive monomers and active dimers toward the dimeric species (12). Activation of the kinase would result from intermolecular interactions.

We have reported that oncogenic p185^{neu} molecules have a greater tendency to oligomerize than protooncogenic p185^{c-neu} (13, 14). Oncogenic p185^{c-neu} receptors also possess higher tyrosine kinase activities than p185^{c-neu} forms (13, 14). A mutation of valine to glutamic acid in the transmembrane region of the p185^{neu} receptor appeared to activate the receptor kinase by inducing aggregation.

Chemical cross-linking (15), nonreducing SDS/PAGE separation (14), sucrose gradients (31), and fluorescent spectroscopic studies (16) support the notion of ligand-induced dimerization of tyrosine kinase receptors. In addition, heterodimer formation of the epidermal growth factor receptors and neu receptors has been shown to lead to the activation of the combined receptor tyrosine kinases (4).

In this study we determined the biochemical basis of p185^{c-neu} holoreceptor activation using the purified ligand.

MATERIALS AND METHODS

Silver Staining. Silver staining of immunoprecipitated baculovirus-expressed, semipurified p185^{c-neu} receptors was carried out using neu receptor-specific monoclonal antibody (7.16.4). After washing and lysing with sample buffer, the

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Abbreviations: NDF, neu-differentiation factor; NAF, neu-activating factor.
*To whom reprint requests should be addressed.

proteins were separated by SDS/12.5% PAGE, and finally the proteins were visualized by silver staining (Bio-Rad).

***In Vitro* Kinase Assay.** Immunoprecipitated and washed $p185^{c-neu}$ receptors were used in the *in vitro* kinase assay with 0.2 μM [γ-^{32}P]ATP (5 μCi; 1 Ci = 37 GBq)/100 μM orthovanadate, as described in ref. 11. Various amounts of conditioned medium derived from ATL-2 cells (percent by volume) were added to the cell lysates prior to the immunoprecipitation and kinase reaction. Kinase reactions were stopped by adding sample lysis buffer containing 1 mM unlabeled ATP. After boiling for 5 min, the proteins were separated by SDS/12.5% PAGE. The autoradiographs were exposed for 3 hr at −70°C.

Tyrosine Phosphorylation. The purification of NAF was identical to the purification reported from ATL-2 cells (11) except that in the final step the active protein was electroeluted, denatured, and renatured from the nonreducing gel. DHFR-G8 (1 × 10^6) cells expressing 3 × 10^5 $p185^{c-neu}$ receptors were treated with or without ligand for 10 min at 37°C. Cell lysates were immunoprecipitated with antibody specific for the neu receptor (7.16.4), separated by SDS/5% PAGE under reducing conditions, and Western blotted with anti-phosphotyrosine antibody (PY20).

Covalent Cross-Linking. DHFR-G8 or M1 cells (1 × 10^6) were incubated with or without ligand for 30 min at 4°C and washed with cold phosphate-buffered saline (PBS). Five milliliters of 5 mM sulfosuccinimidyl suberate in PBS was incubated at 22°C for 30 min. The cross-linking reaction was quenched, and cells were solubilized with PI/RIPA buffer (1% Nonidet P-40/1% deoxycholate/0.1% SDS/0.150 M NaCl/0.01 M sodium phosphate, pH 7.4/1% Trasylol/1 mM phenylmethylsulfonyl fluoride/2 mM EDTA/10 mM sodium pyrophosphate/10 mM iodoacetamide) and immunoprecipitated with antibody 7.16.4. The immunoprecipitate was resolved by SDS/5% PAGE, transferred to nitrocellulose, and immunoblotted with anti-p185 carboxyl-terminal antibodies (anti-NCT).

Sucrose Density Gradient Separation. Baculovirus-expressed protooncogenic and oncogenic neu receptors were purified from lysed insect cell membranes by phosphoagarose column chromatography (17). Linear 5–20% (wt/vol) sucrose gradients (10 ml) were prepared in 50 mM imidazole hydrochloride, pH 7.3/0.1% Triton X-100/0.2 mM EGTA/2 mM benzamidine/1 mM phenylmethylsulfonyl fluoride/aprotinin (10 μg/ml)/10% (wt/vol) glycerol. One and two-tenths micrograms of p185c-neu and 3.6 μg of p185c-neu receptors were sedimented (in a total volume of 0.5 ml, layered top of the gradient) at 38,000 rpm for 20 hr in SW41TI rotor (Sorvall) at 4°C. After sedimentation, 0.5 ml of each fraction was collected and diluted with the above buffer and immunoprecipitated with anti-NCT antisera followed by Western blotting with the same antisera.

^{125}I-Labeled NAF Binding Assay. Purified NAF was radioiodinated using the Bolton–Hunter reagent (NEN). The labeled protein was first purified by G-10 gel filtration. The specific activity was 3.8 × 10^4 cpm per ng. The binding of ^{125}I-labeled NAF was carried out with purified neu receptors expressed in baculovirus-infected insect cells at 4°C for 1 hr; the nonspecific binding was determined by the addition of a 200-fold excess of unlabeled NAF together with labeled NAF. Ligand-bound neu receptors were immunoprecipitated with anti-NCT antisera, and the immunoprecipitated ligand–receptor–protein A bead complex was washed three times with washing buffer. The radioactivity of the immune complex was measured, and the specific binding was obtained by subtracting nonspecific binding from the total binding. Scatchard analysis was performed as described (4).

RESULTS

NAF Effects on Cells. Receptor transactivation may complicate the interpretation of *in vitro* kinase assay results. This process may have obfuscated the studies of NDF (5–7) and heregulin (8). To eliminate transactivation, we have used purified membrane lysates derived from insect SF9 cells expressing $p185^{c-neu}$ (17) to study receptor activation and oligomerization. Purified membrane lysates were immunoprecipitated with the monoclonal anti-receptor antibody (7.16.4). After lysis and SDS/PAGE separation of the proteins, silver staining revealed a single band of 185 kDa (Fig. 1*A*), in addition to the heavy chain of the antibody molecule (50 kDa). The immune complex kinase assays allowed us to compare $p185^{c-neu}$ phosphorylation driven by increasing concentrations of the purified NAF obtained from conditioned medium derived from ATL-2 cells (Fig. 1*B*). These experiments indicated that NAF mediates an increase in $p185^{c-neu}$ phosphorylation in a dose-dependent manner.

NAF Stimulates Receptor Association and Phosphorylation. The ability of NAF to stimulate the *in vivo* tyrosine phosphorylation of the neu receptor on mammalian cells was initially examined. Immunoprecipitation of $p185^{c-neu}$ from NAF-treated DHFR-G8 cells was followed by immunoblotting with anti-phosphotyrosine antibody. The NAF ligand used in these experiments was derived from M1 cells by a purification method identical to that used for ATL-2 NAF;

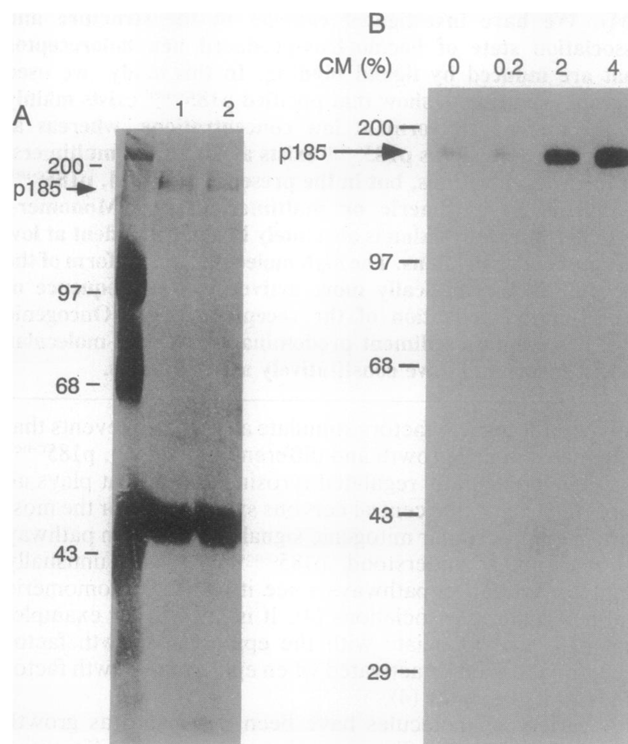

FIG. 1. (*A*) Silver staining of immunoprecipitated baculovirus-expressed $p185^{c-neu}$. Semipurified normal neu receptors were immunoprecipitated with monoclonal anti-neu receptor antibody (7.16.4). After washing and lysing with sample buffer, the proteins were separated by SDS/12.5% PAGE, and finally the bands were visualized by silver staining (Bio-Rad). (*B*) Effect of neu ligand from ATL-2-conditioned medium (CM) on the kinase activity of immunoprecipitated $p185^{c-neu}$ in the *in vitro* kinase assay using 0.2 μM [γ-^{32}P]ATP (5 μCi)/100 μM orthovanadate as described in ref. 11. Various amounts of conditioned medium (percent by volume) were added to the cell lysates prior to immunoprecipitation and the kinase reaction. Kinase reactions were stopped by adding sample lysis buffer containing 1 mM unlabeled ATP. After boiling for 5 min, the proteins were separated by SDS/12.5% PAGE. The autoradiographs were exposed for 3 hr at −70°C.

both NAF preparations have similar size and identical kinase activities (data not shown). The data in Fig. 2*A* showed increased p185^{c-neu} tyrosine phosphorylation that was clearly dependent on the amount of NAF used. As shown in Fig. 2*B*, the cross-linked product obtained after cross-linking with the homobifunctional, noncleavable cross-linker sulfosuccinimidyl suberate in the presence and absence of ligand in DHFR-G8 and M1 cells indicates the presence of 200-kDa and 400-kDa bands corresponding to monomeric and dimeric form of the cross-linked p185^{c-neu} receptor. In addition, the abundance of the dimeric receptor form increases with increasing amounts of the conditioned medium (Fig. 2*B*).

Binding of NAF to Baculovirus-Expressed Holoreceptor. We next examined directly the binding of radiolabeled NAF to purified normal neu receptors expressed in baculovirus (Fig. 2*C*). NAF binding appears to be saturable at a low concentration of radiolabeled ligand. The apparent dissociation constant (K_d) is 1.3 nM, and p185^{c-neu} displays a single high-affinity ligand-binding site.

We next used sucrose gradients to examine the association of purified baculovirus-produced p185^{c-neu} holoreceptor into higher molecular forms in the absence of cross-linkers. Fractions obtained after sucrose gradient ultracentrifugation of the protooncogenic neu receptors were immunoprecipi-

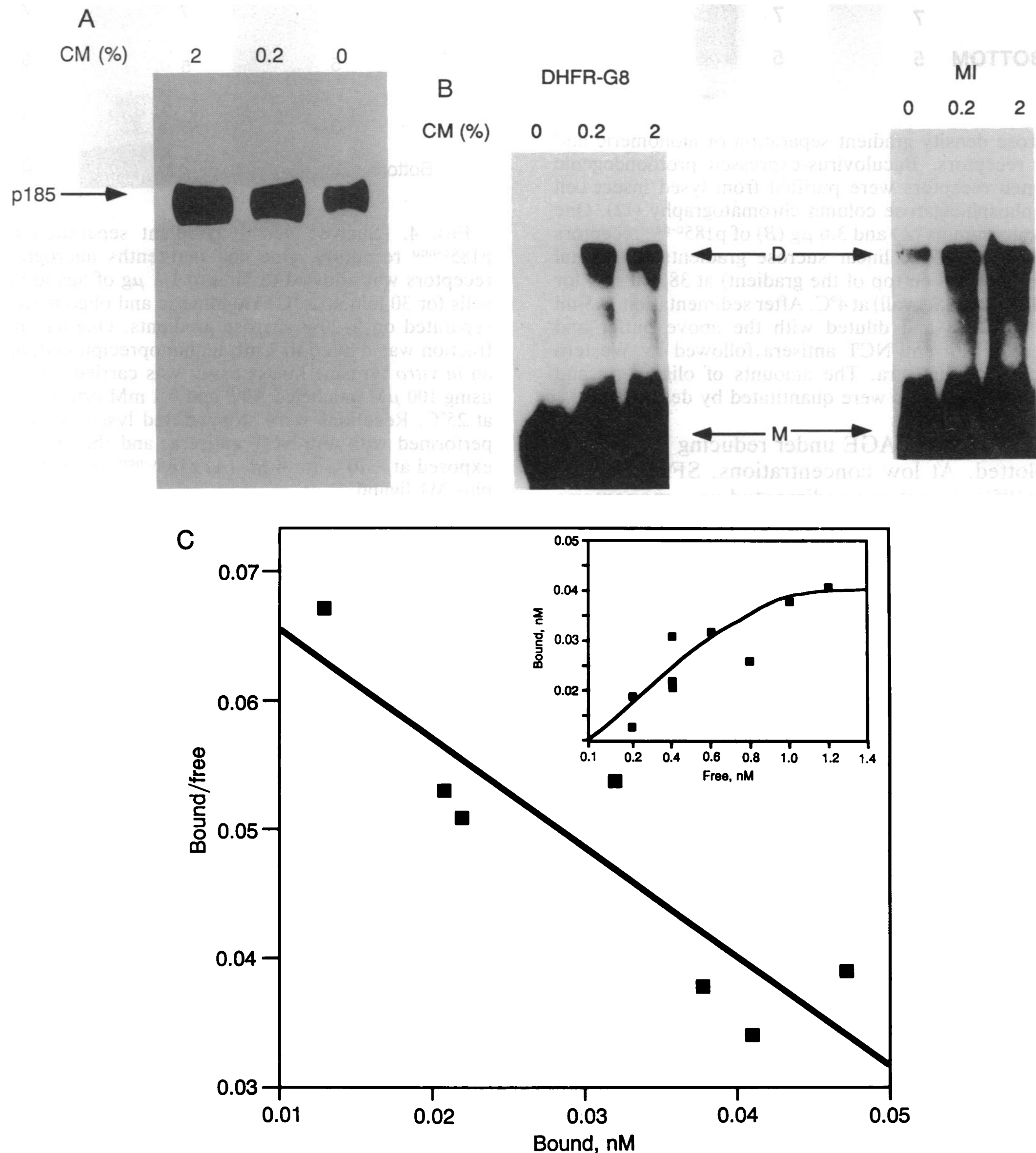

FIG. 2. (*A*) Effect of M1-conditioned medium (CM) on p185^{c-neu} phosphotyrosine content. The purification procedure of NAF from M1 cells was identical to the purification procedure used for ATL-2 cells (11). DHFR-G8 cells (1×10^6) expressing 3×10^5 p185^{c-neu} receptors were treated with or without ligand for 10 min at 37°C. Cell lysates were immunoprecipitated with anti-neu receptor antibody (7.16.4), separated by SDS/5% PAGE under reducing conditions, and Western blotted with anti-phosphotyrosine antibody (PY20). (*B*) Cross-linking of p185^{c-neu} receptors in the presence of ligand. Either DHFR-G8 or M1 cells (1×10^6) were incubated with or without ligand for 30 min at 4°C and washed with cold PBS. Five milliliters of 5 mM sulfosuccinimidyl suberate in PBS was incubated at 22°C for 30 min. The cross-linking reaction was quenched; cells were solubilized with PI/RIPA buffer and immunoprecipitated with 7.16.4. The immunoprecipitate was resolved by SDS/5% PAGE, transferred to nitrocellulose, and immunoblotted with anti-p185 carboxyl-terminal antibodies (anti-NCT). (*Left*) DHFR-G8 cells. (*Right*) M1 cells. Monomers (M) and dimers (D) are shown by arrows. (*C*) Scatchard analysis of binding of radioiodinated NAF to purified p185^{c-neu} receptor. The nonspecific binding was determined by the addition of a 200-fold excess of unlabeled NAF together with labeled ligand. The ligand-bound receptors were immunoprecipitated with anti-NCT antisera, and the immunoprecipitated ligand–receptor–protein A complex was washed three times with washing buffer. The specific binding was calculated by subtracting nonspecific binding from the total binding. Scatchard analysis was performed with binding data.

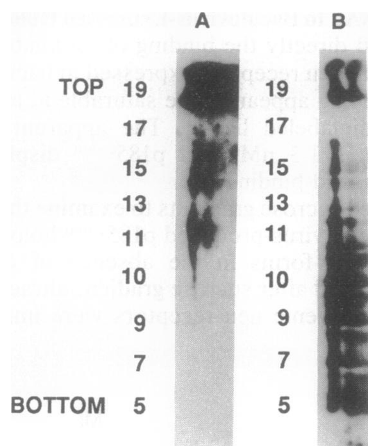

FIG. 3. Sucrose density gradient separation of monomeric and oligomeric neu receptors. Baculovirus-expressed protooncogenic and oncogenic neu receptors were purified from lysed insect cell membranes by phosphoagarose column chromatography (12). One and two-tenths micrograms (*A*) and 3.6 μg (*B*) of $p185^{c-neu}$ receptors were sedimentated in 5–20% linear sucrose gradients (in a total volume of 0.5 ml, layered on top of the gradient) at 38,000 rpm for 20 hr in an SW41TI rotor (Sorvall) at 4°C. After sedimentation, 0.5-ml fractions were collected and diluted with the above buffer and immunoprecipitated with anti-NCT antisera followed by Western blotting with the same antisera. The amounts of oligomeric and monomeric $p185^{c-neu}$ receptors were quantitated by densitometry.

tated, separated by SDS/PAGE under reducing conditions, and immunoblotted. At low concentrations, SF9-produced holoreceptor $p185^{c-neu}$ proteins sedimented as a monomeric form at the top of the gradient. However, at higher concentrations, $p185^{c-neu}$ sedimented to the bottom part of the gradient (Fig. 3). Top and bottom parts of the gradient represent monomeric and oligomeric forms of the receptors, respectively.

Monomeric Holoreceptors Associate into High Molecular Weight Forms. The effect of NAF on monomer–oligomer forms of the $p185^{neu}$ and $p185^{c-neu}$ receptor equilibria was next examined. The physical features of the $p185^{neu}$ and $p185^{c-neu}$ receptors were examined in the presence and absence of ligands. At low concentration, $p185^{c-neu}$ receptors exist at the top of the gradient (Fig. 4*A*), whereas $p185^{neu}$ remained at the bottom part of the gradient (*B*) (17). Similar to the oncogenic neu receptors, the M1 ligand-bound $p185^{c-neu}$ receptors also exist at the bottom part of the gradient (Fig. 4 *B* and *C*). These studies indicate that both ligand binding and the oncogenic mutation (valine → glutamic acid at residue 664 of the neu receptor) caused a similar shift of monomeric to oligomeric forms.

To determine the tyrosine kinase activity of these two forms of the receptors, the immunoblots were stripped and reblotted with anti-phosphotyrosine antibody. We observed that the ligand-induced oligomeric form of the $p185^{c-neu}$ receptor has greater tyrosine kinase activity when compared to that of the monomeric form. As shown in Fig. 5, the intensity of the phosphotyrosine bands of the dimeric form (Fig. 5 *B* and *C*) was much greater than the monomeric form of the receptors (Fig. 5*A*). These results are in agreement with the activation of the *in vitro* kinase activity described above.

DISCUSSION

Several studies have suggested that receptor tyrosine kinases are activated by dimerization resulting from ligand binding to the extracellular ligand-binding domains (12). Dimerization may change the conformation of each of the monomers and promote trans-autophosphorylation (18, 19) through enzymatically active tyrosine kinases. Since receptor dimerization appears essential for many types of receptor activation (33), the formation of so-called dominant-negative dimers with inactive mutant receptor forms (20) may be understood better in the context of the observations presented here.

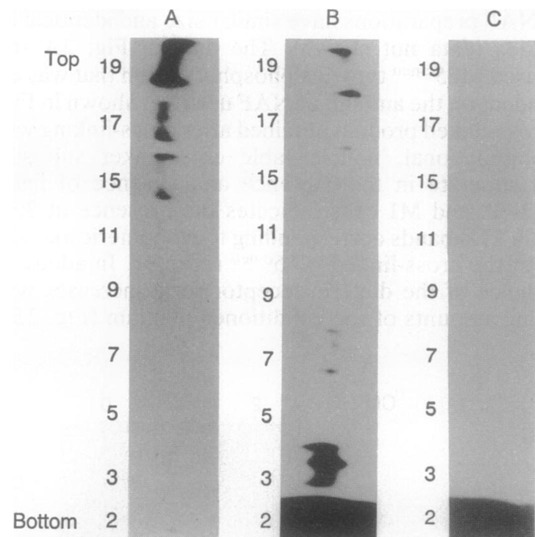

FIG. 4. Sucrose density gradient separation of ligand-bound $p185^{c-neu}$ receptors. One and two-tenths micrograms of $p185^{c-neu}$ receptors was allowed to bind to 1.5 μg of ligand derived from M1 cells for 30 min at 25°C. Monomeric and oligomeric receptors were separated on 5–20% sucrose gradients. One-half milliliter of each fraction was diluted to 1 ml, immunoprecipitated, and washed, and an *in vitro* tyrosine kinase assay was carried out as described (17) using 100 μM unlabeled ATP and 0.1 mM orthovanadate for 20 min at 25°C. Reactions were stopped and lysed, Western blotting was performed with anti-NCT antisera, and the autoradiographs were exposed at −70°C for 4 hr. (*A*) $p185^{c-neu}$. (*B*) $p185^{neu}$. (*C*) $p185^{c-neu}$ plus M1 ligand.

$p185^{c-neu}$ at low molar concentrations exists predominantly in the monomeric form and has only slight tyrosine kinase activity. However, increasing the concentration of $p185^{c-neu}$ (3-fold higher concentration) leads to stable noncovalent intermolecular interactions between the two receptors lead-

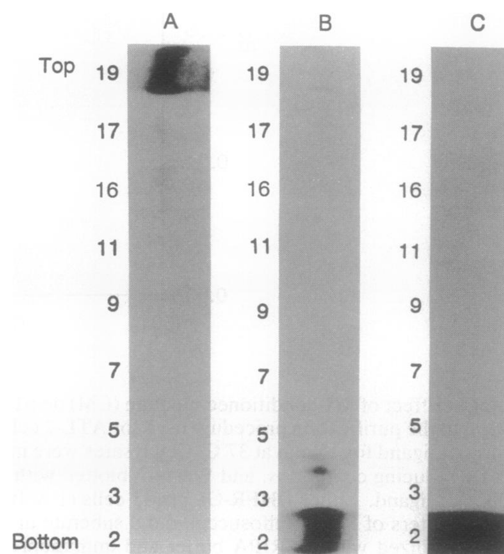

FIG. 5. Tyrosine kinase activity of monomeric and oligomeric neu receptors. The same Western blot as described in Fig. 4 was stripped and reblotted with anti-phosphotyrosine antibody. The autoradiographs were exposed for 4 hr. (*A*) $p185^{c-neu}$. (*B*) $p185^{neu}$. (*C*) $p185^{c-neu}$ plus M1 ligand.

ing to formation of high molecular weight forms. We propose that overexpression of the neu receptor causes the formation of multimeric receptors resulting in constitutive activation that ultimately leads to transformation. Since overexpression of the c-erbB2 protein has been observed in several human cancers (1, 11, 21, 30), these studies provide a physicochemical explanation for the activated forms of amplified receptor species. The conversion of monomeric p185$^{c\text{-}neu}$ to the multimeric form also develops as a consequence of specific binding of the ligand to its extracellular ligand-binding domain of the receptor. It remains to be determined whether each receptor dimer contains one or two molecules of bound ligand.

The solution of the crystal structure of the growth hormone–receptor complex indicated that the ligand receptor interaction occurred between two identical receptor molecules and a single hormone molecule (22). Ligand-induced dimerization and activation of receptor kinases has also been suggested to occur for other receptors such as the platelet-derived growth factor receptor (23, 24). Vascular endothelial growth factor receptors, which belong to the fms-like transmembrane tyrosine kinase family also undergo dimerization in the presence of ligand (25). Dimerization or oligomerization of prolactin receptors is thought to be an obligatory step in signal transduction (26). Cross-linking experiments have indicated the existence of dimeric forms of the receptor for nerve growth factor (27, 28), and receptor aggregation may also play a role in transmembrane signaling of insulin-like growth factor 1 receptors (29). Collectively these studies have shown that ligand-bound holoreceptor proteins on cells associate into high molecular weight forms that are enzymatically active.

With the simple and general system we have described, it will now be possible to ascertain the kinetics of association and the regions of the receptor most relevant to this process. Finally, this system may be adopted to study the extent of heteromer formation of members of this gene family.

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1. King, C. R., Kraus, M. H. & Aaronson, S. A. (1985) *Science* **229**, 974–976.
2. Carpenter, G. & Cohen, S. (1990) *J. Biol. Chem.* **265**, 7709–7712.
3. Kokai, Y., Cohen, A. J., Drebin, J. A. & Greene, M. I. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8498–8501.
4. Wada, T., Qian, X. & Greene, M. I. (1990) *Cell* **61**, 1339–1347.
5. Peles, E., Bacus, S. S., Koski, R. A., Lu, S. H., Wen, D., Ogden, S. G., Levey, R. B. & Yarden, Y. (1992) *Cell* **69**, 205–216.
6. Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Levey, R. B., Koski, R. A., Lu, S. H. & Yarden, Y. (1992) *Cell* **69**, 559–572.
7. Peles, E., Ben-Levy, R., Tzahar, E., Liu, N., Wen, D. & Yarden, Y. (1993) *EMBO J.* **12**, 961–971.
8. Holmes, W. E., Silkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., Sephard, H. M., Xuang, W. J., Wood, W. I., Goeddel, D. V. & Vandlem, R. L. (1992) *Science* **256**, 1205–1210.
9. Lupu, R., Colomer, R., Zugmaier, G., Sarup, J., Shepard, M., Slamon, D. & Lippman, M. E. (1990) *Science* **249**, 1552–1555.
10. Huang, S. S. & Huang, J. S. (1992) *J. Biol. Chem.* **267**, 11508–11512.
11. Dobashi, K., Davis, J. G., Mikami, Y., Freeman, J. K., Hamuro, J. & Greene, M. I. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8582–8586.
12. Yarden, Y. & Schlessinger, J. (1987) *Biochemistry* **26**, 1434–1442.
13. Weiner, D. B., Kokai, Y., Wada, T., Cohen, J. A., Williams, W. V. & Greene, M. I. (1989) *Oncogene* **4**, 1175–1183.
14. Weiner, D. B., Liu, J., Cohen, J. A., Williams, W. V. & Greene, M. I. (1989) *Nature (London)* **339**, 230–231.
15. Fanger, B. O., Stephens, J. E. & Staros, J. V. (1989) *FASEB J.* **3**, 71–75.
16. Carraway, K. L., Koland, J. G. & Cerione, R. A. (1989) *J. Biol. Chem.* **264**, 8699–8707.
17. LeVea, C. M., Myers, J. N., Dougall, W. C., Qian, X. & Greene, M. I. (1993) *Receptor* **3**, 287–303.
18. Honegger, A. M., Kris, R. M., Ullrich, A. & Schlessinger, J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 925–929.
19. Honegger, A. M., Schmidt, A., Ullrich, A. & Schlessinger, J. (1990) *Mol. Cell. Biol.* **1**, 4035–4044.
20. Kashles, O., Yarden, Y., Fischer, R., Ullrich, A. & Schlessinger, J. (1991) *Mol. Cell. Biol.* **11**, 1454–1463.
21. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ulbrich, A. & McGurie, W. L. (1987) *Science* **235**, 177–182.
22. De Vos, A. M., Ultsch, M. & Kossiakoff, A. A. (1992) *Science* **255**, 306–312.
23. Bishayee, S., Majumder, S., Khire, J. & Das, M. (1989) *J. Biol. Chem.* **264**, 11699–11705.
24. Heldin, C. H., Ernlund, A., Rorsman, C. & Ronnstrand, L. (1989) *J. Biol. Chem.* **264**, 8905–8912.
25. Vries, C. D., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N. & Williams, L. T. (1992) *Science* **255**, 989–991.
26. Elberg, G., Kelly, P. A., Djiane, J., Binder, L. & Gertler, A. (1990) *J. Biol. Chem.* **265**, 14770–14776.
27. Grob, P. & Bothwell, M. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 6819–6828.
28. Bradshaw, R. A., Blundell, T. L., Lapatto, R., McDonald, N. Q. & Murray-Rust, J. (1993) *Trends Biochem. Sci.* **18**, 48–52.
29. Ikari, N., Yoshino, H., Moses, A. C. & Flier, J. S. (1988) *Mol. Endocrinol.* **2**, 831–837.
30. Schechter, A. L., Stern, D. F., Vaidyanathan, L., Decker, S. J., Drebin, J. A., Greene, M. I. & Weinberg, R. A. (1984) *Nature (London)* **312**, 513–516.
31. Boni-Schnetzler, M. & Pilch, P. F. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7832–7836.
32. Koland, J. G. & Cerione, R. A. (1988) *J. Biol. Chem.* **263**, 2230–2237.
33. Sternberg, M. J. E. & Gullick, W. J. (1990) *Protein Eng.* **3**, 245–248.