Secondary Dimerization between Members of the Epidermal Growth Factor Receptor Family*

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Growth factor receptors of the epidermal growth factor (EGF) receptor family play pivotal roles in the regulation of cell proliferation and differentiation and are involved in the development of human cancers. It has been well documented that these receptors undergo growth factor-stimulated homo- and heterodimerization as a first step in the initiation of signaling cascades. Here we provide evidence for a new mechanism for growth factor-stimulated receptor dimer formation, designated secondary dimerization. The growth factor-induced dimerization and ensuing receptor trans-autophosphorylation results in the dissociation of the original (primary) receptor dimer. Each phosphorylated receptor monomer then interacts with a new (nonphosphorylated) receptor to form a secondary dimer. Treatment of cells with EGF yields Neu-ErbB2 secondary dimers, and heregulin treatment induces the formation of Neu-EGF receptor (secondary) dimers. The ability of EGF and heregulin to stimulate a cascade of dimerization events points to a novel mechanism by which multiple signaling activities and diverse biological responses are initiated by members of the EGF receptor family.

The interactions of various growth factors and cytokines with cell surface receptor tyrosine kinases initiate a variety of intracellular signaling pathways that when integrated yield cell cycle progression, cell differentiation, or apoptosis. Several classes of receptor tyrosine kinases have been described, among which the EGFR family is of particular interest, because these receptors have been implicated in malignant transformation (1–8). This family includes the epidermal growth factor (EGF)1 receptor (also referred to as ErbB1), the Neu/ErbB2 protein (referred to from here on as Neu), and the more recently identified ErbB3 and ErbB4 proteins (9–12). Two types of ligands interact with members of the EGFR family; EGF is a prototype for ligands that bind to the EGFR, and heregulin (HRG) or Neu differentiation factor represents a family of ligands that bind to both ErbB3 and ErbB4 (10, 13–15). Two of the receptors, EGFR and ErbB4, are capable of ligand-stimulated tyrosine kinase activity. Thus far, the Neu tyrosine kinase is an orphan receptor, whereas ErbB3 appears to be a kinase-defective receptor (14, 16).

The binding of EGF or HRG to their receptors results in receptor dimerization and receptor trans-autophosphorylation. The phosphorylated receptors recruit cellular signaling proteins, through the binding of their Src homology 2 or phosphotyrosine binding domains and thus initiate signaling pathways (17, 18). The binding of EGF stimulates the formation of both EGF homodimers or heterodimers like EGFR-Neu (19–21). Similarly, HRG can stimulate receptor homodimer formation of ErbB3 or of ErbB4 as well as receptor heterodimers like ErbB3 and Neu or ErbB4 and Neu (13, 15). The HRG-promoted formation of these heterodimers provides the molecular basis for the stimulation of Neu tyrosine kinase activity (7, 13, 15, 22).

The mode of heterodimerization between members of the EGFR family may have a significant influence on malignant transformation. For example, the co-expression of ErbB3 and Neu in NIH3T3 cells results in neoplastic transformation, whereas neither the expression of ErbB3 nor Neu alone is sufficient for transformation (1, 7). The heterodimerization of the EGFR with Neu results in cell transformation, whereas the replacement of wild type Neu by its kinase-defective counterpart abrogates transformation (2, 23). Moreover, the expression of Neu in breast and ovarian carcinomas correlates with a poor prognosis (1, 2, 5, 6). The molecular basis for this is likely due to an enhanced tendency of the orphan Neu receptor to form heterodimers with other members of the EGFR family (16, 22, 24).

In this study we present evidence for a new mode of inter-receptor interaction, designated secondary dimerization. Our results show that ligand-induced dimer formation might be followed by dimer dissociation and interaction of the individual, activated receptors with inactive receptors to form new, secondary dimers.

MATERIALS AND METHODS

Cells, Growth Factors, Antibodies, and Inhibitors—Rat pheochromocytoma PC12 cell line 177.2 (25, 26) was used in this study. Recombinant heregulin (rHRG-β177–241) was provided by Dr. Mark Sliwkowski (Genetech, Inc.), and EGF was purchased from Sigma. Anti-EGFR polyclonal antibodies (SC 03) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-c-Neu monoclonal antibodies (Ab-4) were from Calbiochem (San Diego, CA), anti-ErbB3 (2F12) and anti-c-ErbB4 (AB-2) monoclonal antibodies were from Neo Markers (Fremont, CA), and anti-phosphotyrosine monoclonal antibodies (PY20) were from Transduction Laboratories (Lexington, KY). Antibodies were tested for their specificity, and no cross-reactivity was detected. Tyrostatins were purchased from Calbiochem (San Diego, CA).

Cell Growth, Immunoprecipitation, Western Blots, and Determination of Tyrosine Kinase Activity—PC12 cells were grown on 15-cm plastic dishes in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 100 units/ml of penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Amphotericin B (Sigma) in a CO2 incubator (5% CO2) at 37 °C.

Cell stimulation, immunoprecipitation, and Western blotting were carried out as described previously (26). Nearly confluent cell cultures were incubated for 16–20 h in growth medium containing 0.1% serum. Then the cells were gently detached by a short incubation with Hanks’
balanced salt solution (without calcium or magnesium), centrifuged at 500 × g for 5 min, and resuspended in serum-free growth medium. The use of cell suspensions enables the stimulation of a large number of cells in each sample. In control experiments, we did not find a difference between the results obtained when using either attached cells or suspended cells.\(^2\) Cells were dispersed at 1.0 × 10\(^5\) cells/1.5 ml in Eppendorf tubes, and HRG or EGF (20 nM or 100 ng/ml, respectively) was added to some of the tubes for 2 min at 37 °C. Cells subjected to treatment with tyrosine kinase inhibitors were preincubated for 2 min at 37 °C with tyrophostins before the addition of the growth factors. Tyrophostins AG556, AG879, and AG1478 were added to final concentrations of 25, 25, and 0.5 μM, respectively. The incubations were terminated by transferring the tubes to ice, followed by immediate centrifugation to pellet the cells and removal of the medium. The cell pellets were treated with cold lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotonin, 1 μg/ml leupeptin, 1 mM EDTA, 40 mM sodium fluoride, 0.1 mM ammonium molybdate, and 1 mM sodium orthovanadate. After 15 min of incubation on ice, the cell lysates were centrifuged for 10 min at 15,000 × g to remove insoluble material. Antibodies were then added to the lysates, as needed for each experiment, followed by 30 μl of a 50% suspension of protein A-Sepharose (Sigma). The tubes were incubated for 3 h in the cold with slow agitation, and then the immunocomplexes were pelleted by centrifugation, washed once with lysis buffer, and boiled for 3 min in 40 μl of Laemmli sample buffer. The samples were subjected to SDS-polyacrylamide gel electrophoresis (8% acrylamide), and electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Burlington, MA). The blots were blocked by 1 h of incubation with 3% bovine serum albumin in Tris-buffered saline with 0.05% Tween 20, washed, and incubated for 1 h with primary antibodies as indicated. Blots were analyzed by chemiluminescence using horseradish peroxidase-conjugated secondary antibodies and ECL detection reagents (Amersham Corp.).

Determination of tyrosine kinase activity in the immunoprecipitates was performed as described previously (27). Cell growth, cell exposure to either HRG or EGF, cell lysis, and immunoprecipitation were carried out as described above for immunoblotting. Each immunoprecipitate was washed twice with a buffer containing 10 mM HEPES, pH 7.4, 10 mM MgCl\(_2\), 0.1 mM sodium vanadate and resuspended in 60 μl of the same buffer. The assay was initiated by the addition of 20 μl of immunoprecipitate to 20 μl of reaction mixture containing 10 mM HEPES, pH 7.4, 10 mM MgCl\(_2\), 0.1 mM sodium vanadate, and resuspended in 60 μl of the same buffer. The assay was conducted for 10 min at room temperature and were terminated by the addition of 10 μl of 0.5 M EDTA. The tubes were centrifuged, and the supernatants were transferred to Whatman 3MM paper strips, which were incubated overnight in 10% trichloroacetic acid, containing 10 mM sodium pyrophosphate at 4 °C, with gentle agitation. The paper strips were then dried and counted in a scintillation counter.

Assays of tyrosine kinase activity of receptors expressed in insect cells were carried out as described previously (28, 29). Briefly, membranes prepared from insect cells (SF21) preinfected with recombinant viruses (28) harboring either erbB1, neu, or erbB4 (the latter was obtained from K. L. Carraway (Department of Cell Biology Harvard Medical School, Boston, MA)) were incubated for 10 min at room temperature in a 40-μl reaction mixture containing 20 mM HEPES buffer, pH 7.4, 10 mM MgCl\(_2\), 0.5 mM sodium vanadate, 0.5 mg/ml poly-Glu-Tyr\(_{10}\), and 5 μM of [γ-32P]ATP (10 Ci/mmol). The reaction was terminated by the addition of 10 μl of 5 × Laemmli sample buffer (30), and samples were subjected to SDS-polyacrylamide gel electrophoresis (8% acrylamide). Bands of interest were excised from the dried gels, and the associated radioactivity was counted in a scintillation counter (28, 29). Receptors were expressed in insect cells infected with viruses harboring the receptor genes but not in noninfected cells or in cells infected with the wild type virus, as was determined by Western blots.

**RESULTS AND DISCUSSION**

Indications for a new mode of inter-receptor interactions between members of the EGFR family were obtained during a study of the effects of tyrophostins, specific inhibitors of tyrosine kinase activity (31), on receptor dimerization and tyrosine kinase activity in PC12 cells. In these experiments, cells were exposed to EGF or HRG and then subjected to lysis and immunoprecipitation by anti-EGFR or anti-Neu antibodies. The tyrosine kinase activity in the immunoprecipitates was inhibited by the tyrophostin AG556, a specific inhibitor of EGFRI, and by AG879, which specifically inhibits Neu in various cells (27, 31–33), including PC12 cells (34).

The results of these studies suggested that both the EGFRI and Neu tyrosine kinase activities were present in anti-EGFR immunoprecipitates (Table I) and were consistent with previous findings in other cell types (16, 21), as well as studies in PC12 cells,\(^2\) which indicated that EGF treatment induced the formation of EGFRI-Neu heterodimers. This was further reinforced by the data shown in Table I, where the tyrosine kinase activity measured in anti-Neu immunoprecipitates was sensitive to both the EGFRI and Neu antagonists. However, unlike the results obtained with EGFRI and Neu immunoprecipitates from EGF-stimulated PC12 cells, the data obtained from HRG-stimulated cells were unexpected. In particular, immunoprecipitation of Neu from HRG-treated PC12 cells yielded tyrosine kinase activity that was not only sensitive to the Neu kinase inhibitor (AG879) but also to the EGFR kinase inhibitor (AG556) (Table I). This finding indicates the possibility that EGFRI is present in anti-Neu immunoprecipitate, although HRG does not serve as a ligand for EGFRI. Likewise, anti-EGFR immunoprecipitates from HRG-treated PC12 cells showed some sensitivity to the Neu kinase inhibitor (AG879) in addition to the EGFRI kinase inhibitor (AG556) (Table I), indicating the presence of Neu in the immunoprecipitate.

Using insect cell expression systems for members of the EGFRI family (EGFR, Neu, or ErbB4), we have verified that AG879 shows a strong preference for Neu versus EGFRI and is incapable of inhibiting ErbB4 activity (Table II). However, both AG556 and AG1478, which were reported to be specific for the EGFRI (31, 33), are also capable of inhibiting ErbB4 tyrosine kinase activity (Table II).

Thus, one possible explanation for the results obtained with the anti-Neu immunoprecipitates (e.g. Table I) was that HRG stimulated the formation of an ErbB4-Neu primary heterodimer, in which the tyrosine kinase activity of ErbB4 would be sensitive to AG556. Thus far, however, we have not been able to reliably detect the formation of a HRG-stimulated ErbB4-Neu heterodimer in PC12 cells through Western blot analysis, using specific anti-ErbB4 antibodies. An additional possibility, which would not be mutually exclusive with the first, was that HRG stimulated the formation of a Neu-EGFR heterodimer complex. In fact, as shown in Fig. 1 (also, see Fig. 2, below), anti-Neu immunoprecipitates from HRG-treated PC12 cells contain EGFR, as well as Neu and ErbB3.

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\(^2\) D. Gamett, unpublished data.

| Ligand | IP\(^a\) | Tyrophostin | TK\(^b\) |
|--------|-------|-----------|-------|
| EGF    | Anti-EGFR | AG879     | 0.50 ± 0.22 |
|        | AG556   | 0.39 ± 0.15 |
| EGF    | Anti-Neu | AG879     | 0.56 ± 0.15 |
|        | AG556   | 0.53 ± 0.19 |
| HRG    | Anti-Neu | AG879     | 0.52 ± 0.16 |
|        | AG556   | 0.30 ± 0.02 |
| HRG    | Anti-EGFR| AG879     | 0.89 ± 0.04 |
|        | AG556   | 0.57 ± 0.23 |

\(^a\) Immunoprecipitation.

\(^b\) Relative activity of tyrosine kinase, as compared with untreated cells.

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**Secondary Dimerization of EGFR Family Receptors**

**Table I**

*Effect of tyrophostins on tyrosine kinase activity*
Because neither the EGFR nor Neu directly binds HRG, these findings suggest that the EGFR-Neu complex is a secondary outcome of a primary heterodimerization event stimulated by HRG. We suggest that the trans-phosphorylation in the HRG-stimulated ErbB3-Neu dimer might be followed by dimer dissociation and interaction between activated Neu and a latent EGFR. This suggestion is in concert with our previous study, in which we have shown that HRG stimulates the heterodimerization between ErbB3 and Neu in PC12 cells but that following the trans-phosphorylation of ErbB3, it dissociated from Neu and formed a complex with the 85-kDa regulatory subunit (p85) of the phosphatidylinositol 3-kinase (26). Taken together, these results led us to hypothesize that the HRG-stimulated ErbB3-Neu (primary) heterodimer was not stable under conditions of trans-phosphorylation, such that the phosphorylation of the individual receptor (within the primary heterodimer) causes dimer dissociation, thus making the resulting monomers available to interact with other signaling partners. In some cases, the phosphorylated monomeric receptors may bind to Src homology 2 domain-containing proteins like p85. In other cases, however, the phosphorylated monomers can apparently interact with other (nonphosphorylated) receptor monomers to form secondary dimers.

If this hypothesis were correct, we would predict that treatment with different tyrphostins, specific inhibitors of tyrosine kinase activity (31), should have specific effects on the formation of secondary receptor dimers. The data presented in Fig. 2A (lanes 3 and 4) show that this in fact was the case. The treatment of PC12 cells with AG1478, which is a potent and specific antagonist for EGFR in various cells (31, 33, also see Table II), increased the formation of the HRG-stimulated Neu-EGFR secondary dimer, whereas, treatment with AG879, the Neu tyrosine kinase antagonist (31, 33, 34), strongly inhibited the formation of the Neu-EGFR secondary dimer. These findings are fully consistent with the model shown in Fig. 2B.

![Figure 2](http://www.jbc.org/)

**Fig. 2. HRG induces the association of Neu with EGFR.** A, PC12 cells in culture were starved for 1 day in serum-free growth medium, detached, and suspended in serum-free medium. Some cell samples were preincubated for 2 min with tyrphostin AG1478 (0.5 μM) or AG879 (25 μM). Then the cells were stimulated for 2 min with HRG (20 ng/ml) and lysed, and the cell lysates were subjected to immunoprecipitation (IP) using anti-Neu antibodies. The immunoprecipitates were subjected to gel electrophoresis and Western blotting with anti-EGFR antibodies, followed by secondary horseradish peroxidase-conjugated antibodies. The bands were detected by chemiluminescence. B, cell stimulation by HRG results in the formation of the primary heterodimer ErbB3-Neu, followed by trans-phosphorylation, dimer dissociation, and interaction of the phosphate-activated Neu with EGFR to form the secondary heterodimer Neu-EGFR. The tyrphostin AG879 inhibits the tyrosine kinase activity of Neu and thus arrests the dissociation of the primary dimer Neu-ErbB3 and the formation of the secondary dimer Neu-EGFR. The tyrphostin AG1478 inhibits EGFR tyrosine kinase activity and thus arrests the dissociation of the secondary dimer Neu-EGFR. E1, EGFR; B2, Neu; B3, ErbB3. Light gray and dark gray symbols represent low and high phosphorylation levels, respectively. C, the immunoblot (IB) described in A was subjected to a second blotting with anti-phospho-tyrosine antibodies.

Namely, the tyrphostin AG879, by inhibiting Neu tyrosine kinase activity, prevents both the autophosphorylation of Neu and the trans-phosphorylation of ErbB3 and thus prevents the dissociation of the primary Neu-ErbB3 dimer. This, then, inhibits the formation of secondary Neu-EGFR heterodimers. However, tyrphostin AG1478, by inhibiting the EGFR tyrosine kinase activity, prevents the trans-phosphorylation of Neu within the secondary Neu-EGFR dimer, hence inhibiting the dissociation of this dimer, and increases its amount.

**Table II**

| Tyrphostin | EGFR | Neu | ErbB4 |
|-----------|------|-----|-------|
|           | S     | R   |       |
| AG556     | 0.23  | 0.11| 1.21  |
| AG1478    | 0.09  | 0.09| 0.88  |
| AG879     | 0.87  | 0.74| 0.20  |

* Phosphorylation of a substrate (poly-Glu4-Tyr1).

* Receptor autophosphorylation.

**Legend:**
- **P:** Phosphorylation of a substrate (poly-Glu4-Tyr1).
- **IR:** Inhibitor receptor.

**Table II: Effect of tyrphostins on tyrosine kinase activity of receptors expressed in insect cells**

The values in the table represent relative tyrosine kinase activities, as compared with control systems without tyrphostin. Procedures for the determination of the enzymatic activities are described under "Materials and Methods" and in Refs. 28 and 29.
The immunoblot (symbols Neu-ErbB3. dimer EGFR-Neu and thus decrease the levels of the secondary dimer inhibit the trans-phosphorylation and the dissociation of the primary dimer followed by the interaction of activated Neu with ErbB3 to form heterodimer Neu-EGFR. Trans-phosphorylation leads to dimer dissociation catalyzed trans-phosphorylation of Neu is essential for the outcome of a primary EGF-stimulated dimerization between the EGFR and Neu. The formation of this secondary dimer was strongly inhibited by the EGFR antagonist, AG1478, and moderately inhibited by the Neu antagonist, AG879 (Fig. 3A). These results are consistent with the model depicted in Fig. 3B. The marked inhibition of (secondary) Neu-ErbB3 dimer formation by AG1478 suggests that the EGFR-catalyzed trans-phosphorylation of Neu is essential for the dissociation of Neu from the EGFR (within the primary dimer). The modest inhibition of (secondary) Neu-ErbB3 dimer formation by AG879 argues that Neu autophosphorylation and EGFR trans-phosphorylation (by Neu) are less critical for the dissociation of the primary EGFR-Neu dimer. The data presented in Fig. 3C are consistent with the notion that Neu is strongly (trans) phosphorylated by the EGFR within the primary EGFR-Neu heterodimer, as evidenced by almost complete elimination of tyrosine phosphorylation by AG1478. Neu auto-phosphorylation, however, apparently occurs to a lesser extent, because AG879 shows a modest effect. Studies performed with A431 membranes containing EGFR, and insect cell-expressed Neu have also shown that although the EGFR can strongly trans-phosphorylate Neu, there is little or no Neu-catalyzed trans-phosphorylation of the EGFR.

The data presented in Fig. 3D compare the results of immunoprecipitation of ErbB3 with Neu from PC12 cells treated with HRG versus cells treated with EGF. The HRG stimulation would lead to the formation of an ErbB3-Neu (primary) heterodimer, and as previously reported, this yields a doublet in the ErbB3 Western blots that reflected different tyrosine phosphorylation states of ErbB3 (26). The lower mobility (upper band) is exclusively found with p85 (26), leading to the suggestion that this represents a phosphorylated form of ErbB3 that ultimately dissociates from Neu and forms a complex with p85. EGF induces the formation of a Neu-ErbB3 secondary dimer. In this case, the ErbB3 Western blot shows only a single band with a mobility essentially identical to the faster mobility ErbB3 band obtained in HRG-treated cells.

Thus, these results demonstrate that the tyrosine phosphorylation of ErbB3 within Neu-ErbB3 heterodimers differs depending on whether it is a primary Neu-ErbB3 heterodimer or a secondary heterodimer, and presumably these differences will have important consequences regarding the specific substrates that are recruited to ErbB3.

There currently is little debate regarding the fundamental importance of growth factor-stimulated receptor dimer formation in the actions of members of the EGFR family or in the activities of a variety of other receptor tyrosine kinases. Recent studies have shown that receptor activation and dimerization are dependent on the ligand type (24) and that receptor dimerization is not a random process but is subjected to a certain hierarchy (16, 24). The data presented in this study now provide evidence that growth factors can actually stimulate a cascade of receptor dimerization events through a mechanism that reflects the enzyme-substrate nature of the interactions between members of the EGFR family. We propose that growth factor-stimulated receptor dimerization represents a transient rather than a stable interaction. The trans-phosphorylation of one receptor (i.e. the substrate) by its partner receptor (the enzyme) results in the dissociation of the receptor dimer (sim-

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**Fig. 3.** EGF induces the association of Neu with ErbB3. A, PC12 cells in culture were starved for 1 day in serum-free growth medium, detached, and suspended in serum-free medium. The cells were stimulated for 2 min with EGF (100 ng/ml) and then lysed, and the cell lysates were subjected to immunoprecipitation (IP) using anti-Neu antibodies. The immunoprecipitates were subjected to gel electrophoresis and Western blotting with anti-ErbB3 antibodies, followed by secondary horseradish peroxidase-conjugated antibodies. The bands were detected by chemiluminescence. B, EGF induces the formation of the heterodimer Neu-EGFR. Trans-phosphorylation leads to dimer dissociation followed by the interaction of activated Neu with ErbB3 to form the secondary dimer Neu-ErbB3. The tyrosphosphorylated AG1478 and AG879 inhibit the trans-phosphorylation and the dissociation of the primary dimer EGFR-Neu and thus decrease the levels of the secondary dimer Neu-ErbB3. E1, EGFR; B2, Neu; B3, ErbB3. Light gray and dark gray symbols represent low and high phosphorylation levels, respectively. C, the immunoblot (IB) described in A was subjected to a second blotting with anti-phosphotyrosine antibodies. D, cells were stimulated for 2 min with either HRG or EGF (20 nM or 100 ng/ml, respectively). Then the cells were lysed, and the cell lysates were subjected to immunoprecipitation using anti-Neu antibodies. The immunoprecipitates were subjected to gel electrophoresis and Western blotting with anti-ErbB3 antibodies, followed by secondary horseradish peroxidase-conjugated antibodies. The bands were detected by chemiluminescence.

AG879 (Fig. 2C, lane 4), which suggests that Neu autophosphorylation represented only a small percentage of the total tyrosine phosphorylation of Neu.

The data shown in Fig. 3 provide evidence for the formation of another type of secondary dimer. In this case, the stimulation of PC12 cells with EGF led to the formation of a dimer between Neu and ErbB3. Again, because neither of these receptors bind EGF, this dimerization event must be the secondary outcome of a primary EGF-stimulated dimerization between the EGFR and Neu. The formation of this secondary receptor dimer was strongly inhibited by the EGFR antagonist, AG1478, and moderately inhibited by the Neu antagonist, AG879 (Fig. 3A). These results are consistent with the model depicted in Fig. 3B. The marked inhibition of (secondary) Neu-ErbB3 dimer formation by AG1478 suggests that the EGFR-catalyzed trans-phosphorylation of Neu is essential for the dissociation of Neu from the EGFR (within the primary dimer). The modest inhibition of (secondary) Neu-ErbB3 dimer formation by AG879 argues that Neu autophosphorylation and EGFR trans-phosphorylation (by Neu) are less critical for the

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**TABLE III**

Selection of primary and secondary dimers by specific ligands and selective immunoprecipitation

| Ligand | IP* | Primary dimers | Secondary dimers |
|--------|-----|----------------|-----------------|
| HRG    | Anti-Neu | Neu-ErbB3 | EGRF-Neu |
|        |         | Neu-ErbB4 | Neu-Neu |
| HRG    | Anti-EGFR | EGRF-ErbB3 | EGRF-EGFR |
|        |        | EGRF-ErbB4 | EGRF-Neu |
| EGF    | Anti-Neu | EGRF-Neu | Neu-Neu |
|        |         | Neu-ErbB3 | Neu-ErbB4 |
| EGF    | Anti-EGFR | EGRF-EGFR | EGRF-Neu |
|        |        | EGRF-ErbB3 | EGRF-ErbB4 |

* Immunoprecipitation.

3 P. Guy and K. Carraway, unpublished data.
The EGFR family.

The idea that primary receptor dimerization can give rise to secondary dimerization events is supported by various other lines of study. As alluded to above, data obtained from HRG-treated PC12 cells were consistent with the idea that the primary (HRG-stimulated) receptor dimer formed between ErbB3 and Neu was of a transient nature and upon dissociation leads to ErbB3-p85 interactions (26). A study of the reversible dimerization of the EGFR has shown that phosphorylated monomers appeared after receptor dimerization (19). This delayed appearance of phosphorylated EGFR monomers (as compared with the formation of phosphorylated dimers) could be attributed to the dissociation of the EGFR homodimer. Recent studies have also yielded data consistent with the idea that different members of the EGFR family can become phosphorylated and activated in a ligand-independent manner, i.e. in a manner somewhat analogous to the ligand-independent formation of secondary dimerization events between a phosphorylated/activated receptor monomer and a nonphosphorylated/inactive receptor monomer. Specifically, it has recently been shown that both the EGFR and Neu can be phosphorylated and activated (independent of EGF or HRG) by ligands for G protein-coupled receptors, presumably through a mechanism where a Src-like tyrosine kinase (activated downstream from the G protein-coupled receptor) binds and phosphorylates the EGFR or Neu (35).

The secondary dimer formation is also in concert with the findings that Neu is subjected to trans-activation by both EGFR and HRG receptors (36–38) as well as with the concept of lateral signaling within the cell membrane by combinatorial receptor interactions (16).

The ability to undergo both primary and secondary receptor dimerization events would provide a means by which each member of the EGFR family could form a heterogeneous population of phosphorylated receptor species (depending on the types of primary and secondary receptor dimers that are generated), thereby increasing the diversity of signaling pathways that can be initiated by a single growth factor. In addition, the signals induced by secondary receptor dimers will affect cells that have already been subjected to the first wave of signals emanating from the primary receptor dimerization events. Thus, it is conceivable that the second wave of signals might stimulate or arrest and elongate or terminate the effects induced by the first wave of signals. Thus, the mechanism of secondary dimerization provides an additional means for control and for fine tuning of signals initiated by the members of the EGFR family.

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