Differential Signaling by the Epidermal Growth Factor-like Growth Factors Neuregulin-1 and Neuregulin-2

(Received for publication, April 1, 1998, and in revised form, July 7, 1998)

Colleen Sweeney Crovello‡‡, Cary Laï§, Lewis C. Cantley§, and Kermit L. Carraway III¶

From the §Division of Signal Transduction, Beth Israel Deaconess Medical Center and the Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02215 and the ¶Department of Neuropharmacology, Scripps Research Institute, La Jolla, California 92037

The neuregulins comprise a subfamily of epidermal growth factor (EGF)-like molecules encoded by at least three independent genes (1–3). Analysis of NRG1 knockout mice together with the expression patterns of all three known NRG genes suggest that these growth factors contribute to the development and maintenance of the nervous system (4, 5). Knockout and expression studies also point to roles for NRG1 and NRG2 in cardiac development (5, 6), whereas expression and biochemical studies suggest roles for NRG1 in the morphogenesis of mammary tissue and the late stage development of the neuromuscular junction (1, 7). The neuregulins are known to induce a variety of cellular responses depending on cell type, including proliferation, differentiation, survival, and migration (1, 2, 8) and the regulation of gene expression (7, 9, 10).

The neuregulins are produced as transmembrane precursors with mosaic structures (11, 12). The extracellular portion contains several subdomains, including an immunoglobulin-like domain and an EGF-like domain. Neuregulins have large intracellular domains of unknown function. It is thought that during synthesis and routing, active factors are cleaved from full-length precursors to act as diffusible ligands (13). The first two characterized genes, nrg1 and nrg2, give rise to a variety of tissue-specific splice variants (6, 14–19), yielding products containing various combinations of subdomains. NRG1 and NRG2 have at least two splice alternatives in their EGF-like domains, resulting in α and β isoforms of each. These EGF-like domains are sufficient for growth factor receptor binding and activation and are thought to be critical for neuregulin function (11, 20).

The EGF-like growth factors elicit their cellular responses through the ErbB family of receptor tyrosine kinases, which includes the EGF receptor, ErbB2, ErbB3, and ErbB4. Each of these receptors is composed of a large extracellular ligand-binding domain, a single transmembrane domain, and an intracellular portion that contains a protein-tyrosine kinase activity and a carboxyl-terminal tail (21). The extracellular domains are ~40% homologous among the four family members, and the kinase domains display 60–85% homology, whereas the carboxyl-terminal tails are highly divergent. Specific tyrosine residues within the tail regions become phosphorylated in response to ligand binding, creating docking sites for intracellular signaling proteins containing Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains (22, 23).

As suggested above, the neuregulins are capable of eliciting a variety of responses depending on the cellular context. A key question in ErbB-mediated signaling pertains to the biochemical mechanisms involved in specifying the cellular response to the EGF-like ligands. Such studies focus on how the different EGF-like ligands can induce different responses in a single cell type, and how a single EGF-like ligand can induce different responses in different cell types. Since signaling specificity begins with ligand recognition, intense interest has developed in ErbB receptor-ligand and receptor-receptor interactions and the mechanisms of signal propagation across the plasma membrane.

An accumulating body of evidence suggests that each EGF-like growth factor binds with high affinity to specific ErbB receptors, but can activate heterologous receptors via ligand-stimulated receptor heterodimerization and cross-phosphorylation (24). For example, NRG1 binds with high affinity to either...
ErB3 or ErB4 (25–27), but can promote the tyrosine phosphorylation of ErB2 (28). In fact, it appears that the ErB receptors undergo an extensive network of ligand-stimulated receptor-receptor interactions and that the different receptor homo- and heterodimeric combinations can give rise to different cellular responses (29–34). Hence, it is believed that a major mechanism by which the different EGF-like ligands can elicit diverse responses is by preferentially stimulating different pairs of ErB receptors (35). Differences in cellular responses then may arise from the capacity of the various ErB receptors to recruit and activate distinct SH2 domain-containing signaling molecules (20).

Numerous studies indicate that ErB3 and ErB4 are the binding receptors for the α and β forms of both NRG1 and NRG2 (6, 18, 25–27, 36). However, our previous studies suggest that although the β isoforms of NRG1 and NRG2 bind to the same receptors and exhibit ~50% identity in their EGF-like domains, they exhibit different biological potencies toward cultured cells. In the present study, we have examined this further by characterizing NRG-stimulated signaling pathways in two human mammary tumor cell lines, MDA-MB-468 and MDA-MB-453. We find that the two growth factors elicit different cellular responses in the two cell lines and stimulate differences in the recruitment of SH2 and PTB domain-containing proteins to activated receptors. Since each of these cell lines expresses predominantly only two of the known ErB receptors, the preferential heterodimerization model discussed above cannot fully account for differential signaling by the neurogulins. Our results point to another level of growth factor receptor regulation where two different ligands that engage identical receptors elicit distinct responses.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following reagents were purchased as indicated: ECL reagents and protein A-Sepharose CL-4B from Amersham Pharmacia Biotech; glutathione-Sepharose from Sigma; recombinant human EGF from Upstate Biotechnology, Inc.; monoclonal anti-EGF receptor antibody Ab1 from NeoMarkers; monoclonal and affinity-purified rabbit anti-Shc antibodies, monoclonal anti-phospholipase C-γ antibody, affinity-purified rabbit anti-SHP2 antibodies, and recombinant horseradish peroxidase-linked anti-phosphotyrosine antibody RC20 from Transduction Laboratories; affinity-purified rabbit anti-βc and anti-Erb2 antibodies from Santa Cruz Biotechnology; and affinity-purified rabbit anti-STAT3 antibody from New England Biolabs Inc. Monoclonal anti-ErbB2 3E8 precipitating antibody was from Genentech, and affinity-purified rabbit anti-p85 serum (N-SH2) was described previously (37). Rabbit anti-α5β1 serum (N-SH2) was described previously (38).

The extracellular domains of human NRG1α (heregulin-α residues 1–239) (14) and human NRG1β (heregulin-β residues 1–241) (14) were produced in High Five insect cells as described previously (7), purified by heparin affinity, and used in the cell growth and receptor phosphorylation experiments depicted in Fig. 1. Glutathione S-transferase fusion proteins encoding the EGF-like domains of mouse NRG1α (heregulin-β residues 176–246), mouse NRG2α (NRG2α residues 246–330) (6), and mouse NRG2β (NRG2β residues 246–314) (6) were also produced in High Five cells, purified by glutathione affinity as described previously (6), and used in all experiments. The sequences of NRG1β, NRG2α, and NRG2β fused to glutathione S-transferase are illustrated in Fig. 1A. In all assays, the activity of the mouse NRG1β EGF-like domain was identical to that of the human extracellular domain.

**Cell Culture, Immunoprecipitation, and Immunoblotting**—MDA-MB-453 and MDA-MB-468 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37 °C and 5% CO2. Cells were grown to 70–80% confluence and serum-starved in Dulbecco’s modified Eagle’s medium with 0.1% calf serum for 72 h prior to an experiment. The serum starvation was continued for an additional hour at 4 °C. Beads were collected by microcentrifugation and washed three times in 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM Na3VO4, 1 mM NaF, 10 mM β-glycerophosphate, 5 mM tetrasodium pyrophosphate, and 4 μg/ml each aprotinin, leupeptin, and pepstatin. Lysates were cleared by microcentrifugation for 10 min at 4 °C, and supernatants were incubated with the precipitating antibody for 2 h at 4 °C with gentle rocking. Protein A-Sepharose was added to each sample, the suspension was incubated for an additional hour at 4 °C, and beads were collected by microcentrifugation and washed three times in 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM Na3VO4, 1 mM NaF, 10 mM β-glycerophosphate, 0.2 mM phenylmethylsulfonyl fluoride, and 4 μg/ml each aprotinin, leupeptin, and pepstatin. Beads were boiled in Laemmli buffer, and proteins were separated by either 6 or 6–10% gradient SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose.

Blots were cut horizontally into strips of the appropriate ranges for detecting specific signaling proteins. Strips were blocked in 1% bovine serum albumin (for blotting with anti-phosphotyrosine antibody RC20) or 5% Carnation instant milk (for all other antibodies) in Tris-buffered saline with 0.1% Tween 20 (Tris-buffered saline/Tween) for 1 h at room temperature. Blots were incubated with primary antibodies overnight at 4 °C, washed three times with Tris-buffered saline/Tween, and incubated with horseradish peroxidase-linked secondary antibody for 1 h at room temperature. Blots were washed three times with Tris-buffered saline/Tween, and blotted proteins were visualized by enhanced chemiluminescence using x-ray film.

The bands on film were quantified using the Bio-Rad Gel Doc 1000 system with Molecular Analyst software for statistical analysis. For each co-precipitated signaling protein in a given experiment, the amount of association was defined as 100% for a specific growth factor condition (NRG1β treatment in Figs. 2 and 3 and EGF treatment in Fig. 4), and the percentage of protein associated under other conditions was calculated. These percentages were then averaged for three independent experiments, and the means ± S.E. were plotted.

**RESULTS**

**Biological Responses of Tumor Cells to NRG1 and NRG2 Isoforms**—Previous studies have indicated that although NRG1 and NRG2 share significant homology and bind to the same receptors, ErB3 and ErB4, they exhibit distinct biological properties (6, 19). We have explored this further using two human mammary tumor cell lines, MDA-MB-468 and MDA-MB-453. As a starting point, the influence of the α and β isoforms of NRG1 and NRG2 (sequences depicted in Fig. 1A) on the growth and differentiation properties of the two cell lines was examined at receptor-saturating concentrations (30 nM) of the growth factors.

MDA-MB-453 cells do not significantly proliferate in response to the EGF-like growth factors (data not shown), but do show signs of differentiation. 30 nM NRG1β stimulated morphological changes in a significant percentage of these cells, consistent with their differentiation. This factor changed them from a spherical, refractile morphology to a flattened, more tightly adherent appearance (6). 30 nM NRG1α, NRG2α, or NRG2β only weakly promoted this change (Fig. 1B). Under conditions of low serum, MDA-MB-468 cells progress rapidly through the cell cycle, and proliferation cannot be significantly further stimulated by high serum (data not shown) or by EGF-like growth factors (Fig. 1C). Similar to EGF, however, NRG2β...
significantly inhibited the proliferation of these cells as measured by [3H]thymidine uptake, whereas the other neuregulins had little effect. We and others (39) have observed that EGF induces apoptosis in these cells as determined by trypan blue uptake and changes in cell adhesion, but NRG2β did not induce cell death in these assays (data not shown). Hence, despite their presence at saturating concentrations, the neuregulin isoforms induce biological responses to markedly different extents, and the β isoforms of NRG1 and NRG2 exhibit the most potent biological activities toward the cell lines examined here.

The current model for ErbB-mediated signaling suggests that cellular responses to the EGF-like growth factors reflect the activation and tyrosine phosphorylation of the ErbB receptors. To determine whether gross receptor tyrosine phosphorylation might underlie the differences in the observed biological responses, lysates from cells treated with the NRG isoforms were immunoblotted with anti-phosphotyrosine antibodies (Fig. 1D). In MDA-MB-453 cells, 30 nM NRG1α, NRG1β, and NRG2β stimulated the tyrosine phosphorylation of proteins with molecular masses expected for ErbB family members to approximately similar extents, whereas NRG2α stimulated the tyrosine phosphorylation of these proteins to a lesser extent (Fig. 1D, lower panel). In MDA-MB-468 cells, the tyrosine phosphorylation of a 175-kDa protein was stimulated most prominently by 30 nM EGF, followed by NRG2β (Fig. 1D, upper panel). NRG1α, NRG1β, and NRG2α yielded little response. On the other hand, the tyrosine phosphorylation of a 195-kDa protein was stimulated by NRG1α, but was not significantly stimulated by NRG1β or either NRG2 isoform. Hence, whereas growth inhibition of MDA-MB-468 cells correlated with stimulation of the tyrosine phosphorylation of a 175-kDa protein in the ErbB receptor region, no correlation was observed between receptor tyrosine phosphorylation and cellular response in MDA-MB-453 cells.

NRG-stimulated Signaling Pathways in MDA-MB-453 Cells—The observation that MDA-MB-453 cell response did not correlate with gross receptor tyrosine phosphorylation indicated that NRG1β is capable of efficiently triggering signaling pathways not stimulated by the other NRG isoforms. MDA-MB-453 cells express abundant ErbB2 and ErbB3, but little EGF receptor or ErbB4, suggesting that the differential effects of the growth factors on MDA-MB-453 cell morphology might result from differences in signaling pathways directly emanating from these receptors. To test this, we compared the pattern of SH2 and PTB domain-containing proteins that associate

---

**Fig. 1.** Effect of neuregulins on the growth properties of human mammary tumor cells. A, the EGF-like domains of the α and β isoforms of NRG1 and NRG2 used in this study are aligned. Residues in boldface are conserved or invariant in all active EGF-like growth factors. B, MDA-MB-453 cells were treated with the indicated purified growth factors (30 nM), and the percentage of cells that exhibited a flattened morphology was determined microscopically. C, MDA-MB-453 cells were treated with the indicated purified growth factors (30 nM), and the incorporation of [3H]thymidine into DNA was determined. Error bars represent the S.E. of triplicates. D, MDA-MB-468 (upper panel) and MDA-MB-453 (lower cells) cells were stimulated for 5 min at 37 °C with the indicated growth factors (30 nM), and lysates were immunoblotted with anti-phosphotyrosine antibody. The receptor region of the blot is shown. The data depicted in B–D are representative of at least three independent experiments.
with the receptors in response to the highly potent NRG1β and the much weaker NRG2β.

In the experiment shown in Fig. 2A, lysates from growth factor-treated cells were immunoprecipitated with anti-receptor antibodies under conditions optimized for the preservation of noncovalent interactions. Proteins in precipitates were then resolved by gradient SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Filters were cut horizontally into strips of appropriate ranges for detecting associated signaling proteins and blotted with the indicated antibodies. This experiment is representative of three independent experiments. B, co-precipitating proteins from three independent experiments were quantified, and the percent of receptor association for the various conditions was plotted, where the association observed with NRG1β treatment was defined as 100%.

We observed that the tyrosine phosphorylation of both ErbB2 and ErbB3 in MDA-MB-453 cells was markedly stimulated by treatment with either growth factor at 30 nM (Fig. 2A, upper panels) and that the extent of individual receptor tyrosine phosphorylation in MDA-MB-453 cells was similar for both neuregulins. The receptor region was stripped and reprobed with anti-ErbB2 and anti-ErbB3 antibodies, respectively, to confirm similar levels of receptors in each immunoprecipitation (data not shown). Moreover, similar ErbB2 immunoprecipitations were blotted with anti-ErbB3 antibody and vice versa, demonstrating no detectable co-immunoprecipitation of the dimerizing receptor (data not shown). These results corroborate those obtained by blotting cell lysates with antiphosphotyrosine antibodies (Fig. 1D, lower panel) and indicate that the cellular response to the neuregulins is not solely dependent on the overall extent of ErbB2 and ErbB3 tyrosine phosphorylation.

Fig. 2A (four lower left panels) shows the association of four signaling proteins with ErbB2 before and after stimulation with NRG1β or NRG2β. The extent of association of the 85-kDa subunit (p85) of phosphatidylinositol 3-kinase, the phosphotyrosine phosphatase SHP2, the adaptor protein Grb2, and the 46- and 52-kDa forms of the adaptor protein Shc is plotted in Fig. 2B (left panel). We observed that although NRG2β stimulated similar levels of overall ErbB2 tyrosine phosphorylation as NRG1β, it was reproducibly weaker in stimulating the association of each of the examined signaling proteins with that receptor. Fig. 2A (four lower right panels) and Fig. 2B (right panel) show the extent of association of the same four signaling molecules with ErbB3. Surprisingly, the relative efficiency of NRG1β and NRG2β in stimulating the association of these proteins with ErbB3 depended on the specific signaling protein. p85 appeared to be constitutively associated and was not significantly stimulated by either growth factor. NRG2β appeared to be at least as potent as NRG1β in stimulating ErbB3 association with SHP2 and the Shc isoforms, whereas NRG1β was much more potent in stimulating receptor association with Grb2. Although Grb2 is known to couple activated receptors to the mitogen-activated protein kinase pathway, the extent and kinetics of its activation were similar for the two growth factors.
(data not shown), suggesting that pathways other than mitogen-activated protein kinase underlie the differences in NRG1β and NRG2β biological potencies in these cells. However, differences in the recruitment of Grb2 may be relevant for activation of other Grb2-mediated signaling pathways.

These observations indicate that although both NRG1β and NRG2β bind to the ErbB3 receptor and stimulate similar levels of tyrosine phosphorylation of ErbB2 and ErbB3, they elicit distinct patterns of cellular signaling by differentially promoting the association of SH2 and PTB domain-containing signaling proteins with these receptors. It remains to be determined whether the differences in the associations of the specific signaling proteins examined here are responsible for the observed differences in the biological activities of the neuregulins.

**NRG-stimulated Signaling Pathways in MDA-MB-468 Cells**—We have observed a similar phenomenon of differential signaling in response to NRG1β and NRG2β in MDA-MB-468 cells; however, in this case, the differential recruitment of intracellular signaling proteins more closely parallels ligand-stimulated receptor tyrosine phosphorylation. MDA-MB-468 cells overexpress the EGF receptor and express ErbB3 at moderate levels, but lack ErbB2 and ErbB4 expression. Fig. 3 shows an experiment where MDA-MB-468 cells were treated with 30 nM EGF, NRG1β, or NRG2β, and lysates were immunoprecipitated with anti-ErbB3 antibody. Strips were blotted with anti-phosphotyrosine, anti-p85, and anti-Shc antibodies.

We observed that the different ligands stimulated the tyrosine phosphorylation of ErbB3 to different extents, NRG1β being the most efficient, followed by EGF and then NRG2β. This is consistent with the NRG1β-stimulated tyrosine phosphorylation of the 195-kDa band in lysates from these cells (Fig. 1D, upper panel). Again, the blot was stripped and reprobed with anti-ErbB3 antibody to confirm similar receptor levels in precipitates (data not shown). Recruitment of signaling proteins to ErbB3 precisely paralleled the ligand-stimulated tyrosine phosphorylation of the receptor: NRG1β followed by EGF and then NRG2β.

**Fig. 3.** NRG-stimulated association of Shc and phosphatidylinositol 3-kinase with ErbB3 in MDA-MB-468 cells. Cells were treated with 30 nM EGF, NRG1β, or NRG2β for 5 min at 37 °C. A, lysates were immunoprecipitated (IP) with anti-ErbB3 antibody and analyzed as described for Fig. 2. This experiment is representative of three independent experiments. B, co-precipitating proteins from three independent experiments were quantified, and the percent of receptor association for the various conditions was plotted, where the association observed with NRG1β treatment was defined as 100%.

**Fig. 4.** NRG-stimulated association of signaling proteins with the EGF receptor in MDA-MB-468 cells. Cells were treated with 30 nM NRG1β, NRG2β, or EGF for 5 min at 37 °C. A, lysates were immunoprecipitated (IP) with anti-EGF receptor antibody (Anti-EGFR) and analyzed as described for Fig. 2. This experiment is representative of four independent experiments. B, co-precipitating proteins from four independent experiments were quantified, and the percent of receptor association for the various conditions was plotted, where the association observed with EGF treatment was defined as 100%. PLCγ, phospholipase C-γ.
Differential Signaling by Neuregulins

in contrast with its effects on ErbB3, NRG2β significantly stimulated the tyrosine phosphorylation of the 175-kDa EGF receptor in these cells, whereas NRG1β elicited no detectable response (Fig. 4A, upper panel). Ponceau red staining of the filter indicated that similar levels of EGF receptor were present in each precipitation (data not shown). Consistent with the results obtained with lysates from treated cells, EGF stimulated a much more robust response than either of the neuregulins.

Neuregulin-stimulated recruitment of most signaling proteins to the EGF receptor also paralleled the effect of these factors on EGF receptor tyrosine phosphorylation. Fig. 4A (lower five panels) and Fig. 4B demonstrate that NRG2β more efficiently stimulated the recruitment of p52Shc, SHP2, Cbl, and phospholipase C-γ to the EGF receptor than NRG1β. Surprisingly, whereas EGF was at least an order of magnitude more effective in stimulating receptor tyrosine phosphorylation, it was roughly as potent as NRG2β in stimulating the recruitment of these signaling molecules. The only signaling protein that was specifically sensitive to EGF stimulation was STAT3, the association of which with the EGF receptor was not significantly promoted by the neuregulins. Our observations with MDA-MB-468 cells suggest that although NRG1β and NRG2β both bind to ErbB3, they preferentially signal through different receptors, and signaling through the EGF receptor correlates with inhibition of thymidine uptake (Fig. 4C).

The unexpected observation that NRG2β and EGF stimulated similar levels of recruitment of a number of signaling molecules despite a large discrepancy in gross EGF receptor tyrosine phosphorylation raises the possibility that the activation of only a small subset of receptors is necessary for full signal transduction. If true, concentrations of EGF that induce similar levels of EGF receptor tyrosine phosphorylation as NRG2β should stimulate the same level of recruitment of these signaling proteins. To test this, we compared the recruitment of SHP2 and STAT3 to the EGF receptor in response to 30 nM NRG2β and 1 or 30 nM EGF in the same experiment. Fig. 5 shows that treatment of cells with 1 nM EGF stimulated a similar level of EGF receptor tyrosine phosphorylation as did 30 nM NRG2, whereas 30 nM EGF induced at least a 20-fold stronger response. However, the levels of SHP2 recruitment to the receptor were similar under the three conditions, again suggesting that recruitment of intracellular signaling mole-

the mechanism by which the two NRGs define their signaling receptors in these cells is unclear. MDA-MB-468 cells express abundant EGF receptor and moderate levels of ErbB3, but no ErbB2 or ErbB4. Given this receptor complement, homodimers of the EGF receptor or ErbB3 or heterodimers con-
Existing of the EGF receptor and ErbB3 are possible signaling entities. Since ErbB3 lacks an intrinsic kinase activity (41), it is unlikely that ErbB3 homodimers mediate signaling. Our previous observations and unpublished studies using EGF receptor expressed in insect cells, which lack expression of other ErbB receptors, indicate that neither NRG1β nor NRG2β binds to or stimulates the tyrosine phosphorylation of the EGF receptor in the absence of the other ErbB receptors. Hence, it is unlikely that EGF receptor homodimers mediate neuregulin signaling. Therefore, both NRG1β and NRG2β are likely signaling through heterodimers of the EGF receptor with ErbB3. In support of this notion, we have observed that a monoclonal antibody that blocks NRG binding to ErbB3 inhibits NRG1β-stimulated ErbB3 tyrosine phosphorylation and NRG2β-stimulated EGF receptor tyrosine phosphorylation. Therefore, it appears that although EGF receptor–ErbB3 heterodimers are the signaling entities for both NRG1β and NRG2β in MDA-MB-468 cells, diversity is generated by the preferential activation of ErbB3 by NRG1β and of the EGF receptor by NRG2β within the heterodimeric receptor context.

An interesting observation resulting from our studies with the MDA-MB-468 cells concerns correlations between total receptor tyrosine phosphorylation and the efficiency of signaling. For ErbB3, there is a strong correlation. Tyrosine phosphorylation is stimulated strongly by NRG1β, moderately by EGF, and poorly by NRG2β. Likewise, the recruitment of the signaling proteins Shc and phosphatidylinositol 3-kinase follows the same pattern. There is no similar correlation between the total tyrosine phosphorylation of the EGF receptor and the biological or biochemical responses mediated by this receptor. We estimate that EGF induces at least a 20-fold higher degree of EGF receptor tyrosine phosphorylation than does NRG2β. However, the two growth factors inhibit cellular growth to similar extents and stimulate the recruitment of similar levels of some signaling molecules, including phospholipase Cγ, Cbl, SHP2, and Shc, to the EGF receptor.

Two possibilities might account for this effect. First, NRG2β may preferentially and stoichiometrically induce the tyrosine phosphorylation of sites on the total EGF receptor population responsible for mediating its interaction with phospholipase Cγ, Cbl, SHP2, and Shc. In this model, EGF would induce a wider range of receptor tyrosine phosphorylation events. This possibility seems unlikely because ErbB3, which appears to mediate the response of the EGF receptor to NRG2β, is present at limiting quantities and would need to act in a catalytic manner to stimulate the total EGF receptor pool. Alternatively, it is possible that only a subset of the total EGF receptor population is capable of recruiting phospholipase Cγ, Cbl, SHP2, and Shc following treatment with either NRG2β or EGF. This explanation implies that there are subsets of receptors that are predisposed to mediate signaling events by interacting with SH2 and PTB domain-containing intracellular proteins and that remaining receptors may be functionally inert. Hence, the population of EGF receptors that associate with ErbB3 upon EGF binding or those localized to specific domains at the cell surface where signaling molecules reside may be the functionally active species. The overactivation of the EGF receptor in response to saturating EGF may then stimulate the phosphorylation of the receptor at sites responsible for STAT3 recruitment.

The MDA-MB-453 cells exhibit a different response than the MDA-MB-468 cells. NRG1β and NRG2β stimulate the gross tyrosine phosphorylation of the ErbB2 and ErbB3 receptors to similar extents, but the degree to which signaling molecules associate with the receptors differs depending on the stimulating ligand. NRG2β reproducibly stimulated lower levels of ErbB2 association with the SH2 and PTB domain-containing signaling molecules we examined. However, within the same experiment, the degree to which these signaling molecules associated with ErbB3 varied with the ligand presented to the cells. NRG2β stimulated the recruitment of SHP2 and Shc to ErbB3 as efficiently as did NRG1β, but less efficiently stimulated Grb2 recruitment.

Again, the mechanism underlying the differential recruitment is unclear. These cells express abundant levels of ErbB2 and ErbB3 and very low levels of EGF receptor and ErbB4. We have observed that a monoclonal anti-ErbB3 antibody that blocks binding of NRG to ErbB3 abrogates the tyrosine phosphorylation of ErbB2 and ErbB3, stimulated by both NRG1β and NRG2β. Hence, signaling in these cells is undoubtedly mediated by heterodimeric interactions between ErbB2 and ErbB3. One possibility is that the two different growth factors stimulate the same tyrosine phosphorylation events on ErbB2 and ErbB3, but induce different conformational states of the heterodimer such that certain intracellular interactions are sterically precluded. Another possibility is that the two ligands preferentially induce phosphorylation of different tyrosine residues on ErbB2 and ErbB3. Future experiments will address these possibilities.

Acknowledgments—We gratefully acknowledge the expert technical assistance of A. J. Diamonti and Eric Hanson and thank Dr. Stephen Soltoff for critically reviewing the manuscript.

REFERENCES
1. Burden, S., and Yarden, Y. (1997) Neuron 18, 847–855
2. Lemke, G. (1996) Mol. Cell. Neurosci. 7, 247–262
3. Zhang, D., Slawikowski, M. X., Mark, M., Frantz, G., Akita, R., Sun, Y., Hillan, K., Crowley, C., Brush, J., and Godowski, P. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9562–9567
4. Carraway, K. L. III (1996) Bioessays 18, 263–288
5. Meyer, D., and Birchmeier, C. (1995) Nature 378, 386–390
6. Carraway, K. L. III, Weber, J. L., Unger, M. J., Lesedas, J., Yu, N., Gassmann, M., and Lai, C. (1997) Nature 378, 512–516
7. Jo, S. A., Zhu, X., Marchionni, M. A., and Burden, S. J. (1995) Nature 372, 158–161
8. Rio, C., Rieff, H. I., Qi, P., and Corfas, G. (1997) Neuron 19, 49–50
9. Bacus, S. S., Gukovic, A. V., Zelnick, C. B., Chinn, D., Stern, R., Stancovski, I., Polos, E., Ben, B. N., Farbstein, H., Wen, D., Sela, M., and Yarden, Y. (1993) Cancer Res. 53, 5251–5261
10. Ozaki, M., Sassner, M., Yano, R., Lu, H. S., and Buonanno, A. (1997) Nature 389, 691–694
11. Carraway, K. L. III, and Burden, S. J. (1995) Curr. Opin. Neurobiol. 5, 606–612
12. Pelos, E., and Yarden, Y. (1993) Bioessays 15, 815–824
13. Burgess, T. L., Ross, S. L., Qian, Y. X., Brankow, D., and Hu, S. (1995) J. Biol. Chem. 270, 19188–19196
14. Holmes, W. E., Slawikowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yasunara, D., Abadi, N., Raab, H., Lewis, G. D., Shepard, H. M., Kuan, W.-J., Wood, W. I., Goeddel, D. V., and Vanden, R. L. (1992) Science 256, 1205–1210
15. Marchionni, M. A., Goodearl, A. D., Chen, M. S., Bermingham, M. O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Koyabashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, J. B., Huaan, J. T., Totty, N. F., Otsu, M., McBurney, R. M., Waterfield, M. D., Stroobant, P., and Gwynne, D. (1993) Cell 72, 312–318
16. Meyer, D., and Birchmeier, C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1064–1068
17. Meyer, D., Yamai, T., Garratt, A., Riemthaler, S. E., Kane, D., Theill, E. L., and Birchmeier, C. (1997) Development (Camb.) 124, 3575–3586
18. Chang, H., Riese, D. J., II, Gilbert, W., Stern, D. F., and McMahan, U. J. (1997) Nature 387, 509–512
19. Bushfield, S. J., Michnick, D. A., Chickerling, T. W., Revett, T. L., Ma, J., Woolf, E. A., Comrack, C. A., Dassault, B. W., Woolf, J., Goodearl, A. D., and Gearing, D. P. (1997) Mol. Cell. Biol. 17, 4007–4014
20. Carraway, K. L. III, and Cantley, L. C. (1994) Cell 78, 5–8
21. Ullrich, A., and Schlessinger, J. (1990) Cell 61, 203–212
22. Cantley, L. C., Arg, K. R., Carper, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991) Cell 64, 281–302
23. Pawson, T. (1995) Nature 373, 573–580
24. Tzahar, E., Pinkas-Kramarski, R., Meyer, J. D., Klapper, L. N., Arely, I., Levkovitz, G., Shelly, M., Henis, S., Eisenstein, M., Ratzkin, B. J., Sela, M., Andrews, G. C., and Yarden, Y. (1997) EMBO J. 16, 4938–4950
25. Carraway, K. L. III, Slawikowski, M. X., Akita, R., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vanden, R. L., Cantley, L. C., and Cerione, R. A. (1994) J. Biol. Chem. 269, 14305–14306

3 A. J. Diamonti and K. L. Carraway, unpublished observations.
Differential Signaling by Neuregulins

26. Tzahar, E., Levkovitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D., and Yarden, Y. (1994) J. Biol. Chem. 269, 25226–25233

27. Plowman, G. D., Green, J. M., Culouscou, J. M., Carlton, G. W., Rothwell, V. M., and Buckely, S. (1993) Nature 366, 473–475

28. Sliwkowski, M. X., Schaper, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., III (1994) J. Biol. Chem. 269, 14661–14665

29. Riese, D. J., II, Van Raaij, T. M., Plowman, G. D., Andrews, G. C., and Stern, D. F. (1995) Mol. Cell. Biol. 15, 5770–5776R. T.

30. Riese, D. J., II, Bermingham, Y., Van Raaij, T. M., Buckley, S., Plowman, G. D., and Stern, D. F. (1996) Oncogene 12, 345–353R. T.

31. Riese, D. J., II, Kim, E. D., Elenius, K., Buckley, S., Klagsbrun, M., Plowman, G. D., and Stern, D. F. (1996) J. Biol. Chem. 271, 20047–20052

32. Beerli, R. R., and Hynes, N. E. (1996) J. Biol. Chem. 271, 6071–6076

33. Tzahar, E., Waterman, H., Chen, X., Levkovitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J., and Yarden, Y. (1996) Mol. Cell. Biol. 16, 5276–5287

34. Pinkas-Kramarski, R., Shelly, M., Glathe, S., Ratzkin, B. J., and Yarden, Y. (1996) J. Biol. Chem. 271, 19029–19032

35. Riese, D., and Stern, D. F. (1998) Bioessays 20, 41–48

36. Higashiyama, S., Horikawa, M., Yamada, K., Ichino, N., Nakano, N., Nakagawa, T., Miyagawa, J., Matsushita, N., Nagatsu, T., Taniguchi, N., and Ishiguro, H. (1997) J. Biochem. (Tokyo) 122, 675–680

37. Carraway, K. L., III, Soltoff, S. P., Diamonti, A. J., and Cantley, L. C. (1995) J. Biol. Chem. 270, 7111–7116

38. Soltoff, S. P., Carraway, K. L., III, Prigent, S. A., Gillick, W. G., and Cantley, L. C. (1994) Mol. Cell. Biol. 14, 3550–3558

39. Chin, Y. E., Kitagawa, M., Kuida, K., Flavell, R. A., and Fu, X. Y. (1997) Mol. Cell. Biol. 17, 5328–5337

40. Daly, J. M., Jannot, C. B., Beerli, R. R., Graus, P. D., Maurer, F. G., and Hynes, N. E. (1997) Cancer Res. 57, 3804–3811

41. Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A., and Carraway, K. L., III (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8132–8136