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We have engineered a panel of Ba/F3 cell lines that express, singly and in every pairwise combination, the four erbB family receptors. Using this panel of cell lines, we have evaluated hormone-induced erbB family receptor phosphorylation and coupling to downstream signaling proteins and physiologic responses. To date, we have tested six epidermal growth factor (EGF) family hormones: EGF, transforming growth factor alpha (TGFα), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), and neuregulin-beta (NRGβ), also known as heregulin or neu differentiation factor. EGF, BTC, and NRGβ exhibit distinct activities, while EGF, TGFα, and HB-EGF are functionally equivalent. Furthermore, the four erbB family receptors exhibit differential coupling to cellular signaling proteins and physiologic responses. This suggests that cellular responses to activation of the EGF family/erbB family signaling network are specfied by several hierarchical mechanisms.

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Progress Report

I. Introduction

A. Introduction to the problem 3
B. The EGF family/erbB receptor family signaling network 3
C. Experimental approach 5

II. Materials and Methods

A. EGF Family Hormones 6
B. Cell lines and cell culture. 6
C. Plasmid constructions and generation of recombinant Ba/F3 derivatives 6
D. Stimulation and analysis of erbB family receptor tyrosine phosphorylation. 7
E. Stimulation and analysis of IL-3 independent responses 7

III. Body - Assessment of ★Proposal Technical Objectives and ✣Other Experiments

★*A. Subclone erbB receptor and neuregulin cDNAs into retrovirus-based expression vectors and generate recombinant retroviral stocks to facilitate gene transfer and expression* 7
★*B. Develop cultured cell systems for expression and purification of recombinant neuregulin* 7
★*C. Determine what erbB receptors are neuregulin effectors in Ba/F3 cells* 8
★*D. Determine if neuregulin acts as an adhesion molecule of receptor for erbB proteins* 9
★*E. Assess effector-specific or presentation specific neuregulin-induced protein tyrosine phosphorylation* 9
✣*F. Identify the patterns of erbB receptor tyrosine phosphorylation stimulated by other EGF family hormones* 9
★✣*G. Determine if hormone-induced erbB receptor signaling confers IL-3 independent growth to Ba/F3 cells* 10
✣ *H. Identify cellular signaling proteins differentially coupled to each erbB family receptor* 11
★*I. Determine if neuregulin-induced erbB receptor signaling is capable of transforming the growth or altering the differentiative capacity of MCF-10 cells* 12
✣*J. Characterize the responses of the MCF-10A cell line to erbB4 signaling* 12

IV Conclusions 14

V. References 23

VI. Appendices

A. Figures and Tables

Figure 1. Patterns of Hormone-induced ErbB Family Receptor Phosphorylation 26

Table 1. Physiologic Responses to ErbB Family Receptor Activation 26

Figure 2. Immunoprecipitation and Immunoblotting of Lysates From EGFR+ErbB4 Cells Stimulated with Betacellulin 27

B. Bibliography of publications and abstracts 28

C. Personnel 28

D. Graduate degrees resulting from contract support 28

I. Introduction

A. Introduction to the problem

Breast cancer is one of the leading causes of cancer death in women in the United States; approximately 46,000 American women died of the disease in 1993 [Boring, *et al.*, 1993]. One model is that breast cancer results from disruptions in the normal hormonal regulation of mammary gland epithelial cell proliferation and differentiation. We have undertaken experiments to characterize the signaling network composed of the epidermal growth factor (EGF) family of peptide hormones and the erbB family of receptor protein tyrosine kinases. We also have begun experiments that assess the effects of this network on the proliferation, differentiation, and malignant growth transformation of mammary epithelial cells Therefore, these experiments are beginning to shed light on the role that these proteins play in breast carcinogenesis.

B. The EGF family/erbB receptor family signaling network

Deregulated signaling by the four receptor tyrosine kinases encoded by the erbB gene family (erbB-1/epidermal growth factor receptor [EGFR], neu/erbB-2/HER2 , erbB-3/HER3 , and erbB-4/HER4), has been implicated in a number of human cancers, including mammary cancer, ovarian cancer, gastric cancer, and glioblastoma [Reviewed in Hynes and Stern, 1994]. Understanding the normal and pathological functions of these receptors requires that their regulation by hormones be elucidated. However, there are at least 15 different agonists for erbB family receptors, including epidermal growth factor (EGF), transforming growth factor alpha (TGFα), amphiregulin (AR), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), cripto (CR), epiregulin (EPI), and the several differentially spliced variants of the neuregulins (NRGs), also known as gp30, heregulins, neu

differentiation factors, glial growth factors, and acetylcholine receptor inducing activity [Reviewed in Groenen, *et al.*, 1994]. While a single erbB family receptor can in some cases bind more than one ligand (e.g. EGFR) [Riese, *et al.*, in press], a single EGF family hormone can in some cases bind to multiple erbB family receptors (e.g. BTC) [Riese, *et al.*, 1996]. Furthermore, while an erbB family receptor expressed by itself may not be stimulated by a given EGF family hormone, this receptor can be activated when co-expressed with another erbB family receptor that can bind the hormone [Reviewed in Earp, *et al.*, 1995]. For example, EGF does not bind or activate neu expressed on its own, but activates neu when coexpressed with the EGFR [Akiyama, *et al.*, 1988; King, *et al.*, 1988; Stern and Kamps, 1988; Connelly and Stern, 1990]. This "transmodulation" activation of neu by EGFR apparently occurs through the formation of EGF-driven receptor heterodimers and receptor cross-phosphorylation [Goldman, *et al.*, 1990; Wada, *et al.*, 1990; Qian, *et al.*, 1992; Spivak-Kroizman, *et al.*, 1992].

The physiological responses to agonists for erbB family receptors depends on their ability to coordinately activate multiple receptors that are differentially expressed and have different signaling capabilities. Although NRGs were initially purified by their ability to induce neu tyrosine phosphorylation, and were thought to be ligands for neu, NRG does not bind neu and/or induce neu tyrosine phosphorylation in a variety of cell types or in solution [Culouscou, *et al.*, 1993; Peles, *et al.*, 1993; Plowman, *et al.*, 1993b; Sliwkowski, *et al.*, 1994; Tzahar, *et al.*, 1994]. Instead, NRG binds erbB-3 [Carraway, *et al.*, 1994; Kita, *et al.*, 1994; Sliwkowski, *et al.*, 1994; Tzahar, *et al.*, 1994] and erbB-4 [Plowman, *et al.*, 1993a; Plowman, *et al.*, 1993b; Culouscou, *et al.*, 1993]. Co-expression of erbB-3 or erbB-4 with neu permits NRG-induced tyrosine phosphorylation of neu, presumably through the formation of neu/erbB-3 or neu/erbB-4 heterodimers [Plowman, *et al.*, 1993b; Carraway, *et al.*,

1994; Kita, *et al.*, 1994; Sliwkowski, *et al.*, 1994; Riese, *et al.*, 1995]. Despite the many combinatorial possibilities afforded by assortment of four different receptors, interactions of erbB family members with their agonists have only been investigated in a piecemeal fashion. Receptors of different species origins have been mixed in gene transfer experiments, only a subset of receptor combinations have been tested, and interpretation is hampered by the variety of cell backgrounds used and in many cases by the failure to determine the endogenous erbB family receptor expression in the cell lines used. Finally, the hormone-regulated coupling of different erbB family receptors and combinations of receptors to different downstream signaling pathways has not been systematically investigated for any EGF family agonist.

C. Experimental approach

In order to address these issues we have undertaken a parallel analysis of the aggregate signaling potential of the erbB receptor family. We have expressed all four human erbB family receptors, singly and in each pairwise combination, in the interleukin-3-dependent mouse Ba/F3 pro-B-lymphocyte cell line. By assessing erbB receptor tyrosine phosphorylation in the resulting panel of cell lines following stimulation with EGF family hormones, we have performed the first comprehensive evaluation of ligand-induced erbB family receptor activation. Furthermore, by assessing the induction of IL-3-independent growth in this panel of cell lines following hormonal stimulation, we have also evaluated ligand-induced erbB family receptor coupling to cellular signaling pathways. These data establish that different EGF family hormones stimulate distinct patterns of erbB receptor phosphorylation and coupling to cellular signaling pathways. Moreover, these data suggest several additional mechanisms by which biological responses are specified by interactions among erbB family receptors and their agonists.

II. Materials and Methods

A. EGF Family Hormones

The production and/or synthesis of recombinant AR, NRGβ, and BTC have been described [Riese, *et al.*, 1995; Riese, *et al.*, 1996; Riese, *et al.*, in press]. Recombinant EGF and TGFα were obtained from Collaborative Biomedical. Recombinant AR, BTC and NRGα were obtained from R&D Systems. Recombinant EPI has been provided on a collaborative basis from Taisho Pharmaceuticals, Tokyo, Japan. CR peptide has been provided on a collaborative basis from William J. Gullick (ICRF) and David S. Salomon (NCI/NIH).

B. Cell lines and cell culture.

The Ba/F3 mouse pro-B-lymphocyte cell line [Palacios and Steinmetz, 1985] and its derivatives were grown in RPMI (Gibco/BRL) supplemented with 10% fetal calf serum (Sigma) and Interleukin-3 (IL-3) supplied as 10% conditioned medium from the WEHI-3B mouse myelomonocytic leukemia cell line [Daley and Baltimore, 1988]. Ba/F3 derivatives transformed with constructs expressing erbB family receptors were grown in medium supplemented with 200ug/ml G418 (Gibco/BRL).

C. Plasmid constructions and generation of recombinant Ba/F3 derivatives.

The construction of recombinant retroviral vectors expressing the four erbB family receptor cDNAs have been described previously [Riese, *et. al.*, 1995]. The generation and characterization of Ba/F3 derivatives expressing the four erbB family receptors, singly and in every pairwise combination, have also been described previously [Riese, *et al.*, 1995].

D. Stimulation and analysis of erbB family receptor tyrosine phosphorylation.

The stimulation and analysis of erbB family receptor tyrosine phosphorylation have been described earlier [Riese, *et al.*, 1995].

E. Stimulation and analysis of IL-3 independent responses.

The stimulation and analysis of IL-3 independent responses have been described earlier [Riese, *et al.*, 1995].

III. Body - Assessment of Proposal Technical Objectives and Other Experiments

A. Subclone erbB receptor and neuregulin cDNAs into retrovirus-based expression vectors and generate recombinant retroviral stocks to facilitate gene transfer and expression.

We have subcloned the four different erbB family receptor cDNAs into the pLXSN recombinant retrovirus-based expression vector [Riese, *et al.*, 1995]. We have also subcloned the neuregulin-α cDNA into this same vector [Riese and Stern, unpublished data]. While we have packaged these constructs into recombinant retroviral stocks, these stocks were not used to generate the Ba/F3 derivatives described elsewhere in this report.

B. Develop cultured cell systems for expression and purification of recombinant neuregulin.

We have introduced a neuregulin-α (NRGα) expression vector into mouse C127 fibroblasts, generating cell lines that stably express and secrete NRGα. We have also engineered recombinant baculovirus stocks containing a NRGα cDNA. However, we have not conclusively determined that infection of insect cells with these stocks results in the production of biologically active NRGα. Dr. Frank Jones,

a postdoctoral fellow in the Stern Laboratory, is continuing these efforts to produce biologically active recombinant NRG.

To bypass these difficulties in producing recombinant NRG, we established a collaboration with Drs. James D. Moyer, Brad C. Guarino, and Glenn C. Andrews, Pfizer Central Research, Groton, CT. They have supplied us with NRGβ as a refolded, biologically active, chemically-synthesized 65-mer peptide corresponding to amino acids 177 to 241 of the NRG β1 isoform [Riese, *et al.*, 1995; Barbacci, *et al.*, 1995]. We also established a collaboration with Drs. Sharon Buckley and Gregory D. Plowman, Sugen, Inc., Redwood City, CA. They have supplied us with human recombinant betacellulin (BTC) and human recombinant amphiregulin (AR) as refolded, biologically active peptides expressed in *E. coli.* We also established collaborations with Dr. Michael Klagsbrun (Children's Hospital, Boston, MA), who is supplying us with recombinant heparin-binding EGF-like growth factor (HB-EGF), with Dr. David Salomon (NIH/NCI) and Dr. William J. Gullick (ICRF), who are supplying us with cripto peptide, and with Taisho Pharmaceuticals, Tokyo, who is supplying us with recombinant epiregulin. We have purchased recombinant transforming growth factor alpha (TGFα) and EGF from Collaborative Biomedical, and recombinant AR, NRGα, and BTC from R&D Systems.

C. Determine what erbB receptors are neuregulin effectors in Ba/F3 cells

As described previously, neuregulin-β (NRGβ) stimulates erbB-4 tyrosine phosphorylation as well as erbB-3 tyrosine phosphorylation, but activates erbB-3 only when erbB-3 is coexpressed with another erbB family receptor. Furthermore, NRG-β stimulates EGFR and neu tyrosine phosphorylation when these receptors are coexpressed with either erbB-3 or erbB-4 [Riese, *et al.*, 1995] (Figure 1).

D. Determine if neuregulin acts as an adhesion molecule or receptor for erbB proteins.

These experiments are being performed by Jonathan McMenamin-Balano, a predoctoral student in the Stern Laboratory. Preliminary evidence suggests that the membrane-bound, immature form of neuregulin acts as a receptor for erbB3 or erbB4 and that the cytoplasmic domain of neuregulin is coupled to cellular signaling pathways.

E. Assess effector-specific or presentation specific neuregulin-induced protein tyrosine phosphorylation.

These experiments are being performed by Jonathan McMenamin-Balano, a predoctoral student in the Stern Laboratory. Preliminary evidence suggests that soluble and membrane bound forms of neuregulin stimulate identical patterns of erbB family receptor tyrosine phosphorylation.

F. Identify the patterns of erbB receptor tyrosine phosphorylation stimulated by other EGF family hormones.

As summarized in Figure 1, EGF, AR, TGFα, and HB-EGF stimulate EGFR tyrosine phosphorylation, as well as the phosphorylation of any other erbB family receptor when it is coexpressed with the EGFR [Riese, *et al.*, 1996; Riese, *et al.*, in press]. Surprisingly, betacellulin stimulates both EGFR and erbB-4 tyrosine phosphorylation. Furthermore, betacellulin stimulates both neu and erbB-3 tyrosine phosphorylation when these receptors are coexpressed with the EGFR. In contrast, betacellulin stimulates neu but not erbB-3 tyrosine phosphorylation when these receptors are coexpressed with erbB-4 [Riese, *et al.*, 1996]. Therefore, NRG-β, EGF, and betacellulin all stimulate distinct patterns of erbB family receptor tyrosine phosphorylation, which may in part account for their differential biological activities.

G. Determine if hormone-induced erbB receptor signaling confers IL-3 independent growth to Ba/F3 cells.

While NRG-β, betacellulin, and EGF can all stimulate the tyrosine phosphorylation of all four erbB family receptors, either directly or through transmodulation (see previous section), activation of different receptors or combinations of receptors may specify unique biological responses through coupling of each receptor to distinct cellular signaling pathways. We investigated this possibility by determining whether ligand stimulation enabled survival or growth of the various Ba/F3 derivatives independent of interleukin-3 (IL-3). Ectopic expression of a number of receptors in Ba/F3 cells permits receptor regulation by the cognate ligands, which in some cases relieves dependence on IL-3 for survival or growth: expression of the erythropoietin receptor with Friend Spleen Focus-Forming Virus gp55 permits IL-3-independent proliferation [Li, *et al.*, 1990]. Similarly, EGF stimulation of Ba/F3 cells expressing exogenous EGFR results in EGFR tyrosine phosphorylation and increased cellular DNA synthesis [Collins, *et al.*, 1988; Shibuya, *et al.*, 1990], while stimulation of Ba/F3 derivatives expressing exogenous platelet derived growth factor (PDGF) receptor with PDGF results in receptor tyrosine phosphorylation and IL-3-independent proliferation [Satoh, *et al.*, 1993].

In the absence of ligand, all of the Ba/F3 derivatives remained dependent on IL-3 for survival, even those lines that display substantial basal receptor tyrosine phosphorylation [Riese, *et al.*, 1995]. Activation of either EGFR or neu in the single recombinant cell lines was associated with IL-3 independent survival but not proliferation, while activation of erbB-3 or erbB-4 in the single recombinants had no biological effect [Riese, *et al.*, 1995; Riese, *et al.*, 1996]. Therefore, ligand stimulation

of erbB phosphorylation was necessary, but not sufficient, for an IL-3 independent response (Table 1).

We also assessed ligand activity in the double recombinant Ba/F3 cell lines. As expected from the responses of the single recombinant cell lines, receptor activation in cells expressing EGFR or neu conferred, with one notable exception, a minimal response of IL-3 independent survival. For example, in EGFR + neu (1+2), EGFR + erbB-4 (1+4), neu + erbB-3 (2+3), and neu + erbB-4 (2+4) cell lines, receptor activation stimulated a minimum of IL-3-independent survival, while in erbB-3 + erbB-4 (3+4) cells none of the ligands stimulated an IL-3 independent response [Riese, *et al.*, 1995; Riese, *et al.*, 1996; Riese, *et al.*, in press]. The exception is the response of 1+3 cells to ligand stimulation. As predicted, betacellulin and EGF stimulated the IL-3 independent survival of 1+3 cells; however, NRG-β failed to stimulate an IL-3 independent response [Riese, *et al.*, 1995; Riese, *et al.*, 1996].

In some of the double recombinant cell lines ligand stimulation of coupling of multiple receptors to signaling pathways acted in a non-additive manner to stimulate an IL-3 independent response (Table 1). In 1+2 cells betacellulin and EGF stimulated IL-3 independent proliferation, while in 1+4 cells EGF, betacellulin and NRG-β stimulated IL-3 independent proliferation [Riese, *et al.*, 1995; Riese, *et al.*, 1996; Riese *et al.*, in press]. Therefore, while activation of either EGFR alone or neu alone stimulated IL-3 independent survival, activation of EGFR along with either neu or erbB-4 conferred IL-3 independent proliferation (Table 1).

H. Identify cellular signaling proteins differentially coupled to each erbB family receptor.

We have previously established the four erbB family receptor stimulate different physiologic responses in Ba/F3 cells (Table 1). This suggests that each receptor is coupled to a distinct set of cellular signaling proteins. We have

confirmed this hypothesis by identifying cellular signaling proteins that are differentially coupled to the four erbB family receptors. In Ba/F3 cells, EGFR activation is accompanied by the phosphorylation of c-Cbl and Shc, two known signaling effectors for EGFR (data not shown). In contrast, activated erbB-4 is not accompanied by c-Cbl or Shc phosphorylation (data not shown). This suggests that Shc and c-Cbl are downstream signaling effectors for EGFR and not for erbB-4. In cells expressing both EGFR and erbB-4, BTC stimulate high levels of EGFR, erbB-4, Shc, and c-Cbl phosphorylation. However, Shc and c-Cbl preferentially complex with EGFR and not with erbB-4, suggesting again that Shc and c-Cbl are signaling effectors for EGFR and not for erbB-4 (Figure 2). Currently we are performing gene transfer experiments with a dominant negative Shc allele and constitutively active Cbl alleles to determine if activation of Shc and/or Cbl is required for the physiological responses of Ba/F3 cells to EGFR activation.

I. Determine if neuregulin-induced erbB receptor signaling is capable of transforming the growth or altering the differentiative capacity of MCF-10A cells.

We are currently establishing experimental conditions for this experiment. See the section below for additional information about the effects of erbB family receptor signaling on MCF-10A cells.

J. Characterize the responses of the MCF-10A cell line to erbB4 signaling.

There is mounting evidence that while activation of either EGFR or neu stimulates mammary cell proliferation and promotes tumorigenesis, increased erbB4 signaling may inhibit proliferation or tumorigenesis by stimulating differentiation. Ectopic treatment of breast tumor cell lines with NRG inhibits their growth and stimulates milk protein synthesis [Peles, *et al.*, 1992; Wen, *et al.*, 1992].

and NRG promotes the differentiation of breast cells *in vivo* [Krane and Leder, 1996; Jones, *et al.*, 1996]. Moreover, erbB4 overexpression in human mammary tumor samples correlates with markers for a more favorable prognosis, suggesting that erbB4 signaling may inhibit tumorigenicity [Bacus, *et al.*, 1996].

We wished to examine the effects of increased erbB4 signaling on the proliferation of MCF-10A cells. However, in these cells erbB4 tyrosine phosphorylation is not stimulated by either NRG [Beerli, *et al.*, 1995] or BTC (data not shown). Therefore, we tried to establish MCF-10A derivatives that ectopically overexpress erbB4 through infection with a recombinant retrovirus containing the neomycin resistance gene (neo^R) and the human erbB4 cDNA (see section II.C.). We noted that this retrovirus stock had an unusally low titer in MCF-10A cells and that the rare transformants that arose from infections with the erbB4 retrovirus did not express higher levels of erbB4 than the parental MCF-10A cells (data not shown). A more careful analysis demonstrates that the low titer of the erbB4 retrovirus stock is specific for erbB4 (Table 1), suggesting that erbB4 is a growth suppressor in breast cells through coupling to cellular differentiation. Future experiments will examine the activity of erbB4 mutants lacking cytoplasmic tyrosine residues that serve as sites for autophosphorylation and docking to receptor effector proteins. These experiments will identify signaling effectors required for the growth inhibitor effect of erbB4 signaling in MCF-10A cells.

IV. Conclusions/Discussion

We have analyzed the hormone-dependent responses conferred upon the Ba/F3 mouse pro-B-lymphocyte cell line by expression of the four erbB family receptors, singly and in pairwise combinations. In the presence of appropriate co-receptors, NRG regulated the tyrosine phosphorylation of all four erbB family receptors. While some of the NRG-induced interactions between erbB family receptors observed had been predicted from previous work, we demonstrate here for the first time that in the presence of erbB-3 or erbB-4, NRG regulates tyrosine phosphorylation of the EGFR, and that the presence of the EGFR, neu, or erbB-4 enables NRG to regulate tyrosine phosphorylation of erbB3. Furthermore, NRG induces IL-3-independent survival or proliferation in only a subset of the lines that exhibit NRG-induced receptor tyrosine phosphorylation. Thus the biological responses to NRG are specified at several different levels of regulation.

Previous analyses of NRG-induced signaling by erbB family receptors have been carried out with a few receptor combinations in a variety of cell backgrounds. In mammary cells, NRGs induce neu tyrosine phosphorylation, can be cross-linked to neu, and binding is increased by neu overexpression [Peles, *et al.*, 1993], at first suggesting that neu is a receptor for NRG. However, NRG fails to induce neu tyrosine phosphorylation and/or bind neu when neu is expressed in fibroblasts, ovarian cells [Peles, *et al.*, 1993], CHO cells [Culouscou, *et al.*, 1993; Plowman, *et al*, 1993b], T-lymphoid cells [Plowman, *et al.*, 1993b], or COS-7 cells [Sliwokowski, *et al.*, 1994], and NRG does not bind to solubilized neu extracellular domains [Tzahar, *et al.*, 1994]. Moreover, NRG binds erbB-3 [Carraway, *et al.*, 1994; Kita, *et al.*, 1994; Sliwkowski, *et al.*, 1994; Tzahar, *et al.*, 1994] or erbB-4 [Culouscou, *et al.*, 1993; Plowman, *et al.*, 1993a; Plowman *et al.*, 1993b; Tzahar, *et al.*, 1994], and co-expression

of erbB-3 or erbB-4 with neu confers NRG responsiveness upon neu, probably through the formation of neu/erbB-3 or neu/erbB-4 heterodimers [Carraway, *et al.*, 1994; Kita, *et al.*, 1994; Plowman *et al.*, 1993b; Sliwkowski, *et al.*, 1994]. This has led to the general working hypothesis that activation of neu by NRG requires the presence of erbB-3 or erbB-4.

The present data are compatible with this conclusion, and extend the model to include NRG regulation of the EGFR. The EGFR and erbB-3 alone fail to respond to NRG for two different reasons. The EGFR does not bind NRG [Holmes, *et al.*, 1992], whereas erbB-3 binds, but is impaired for kinase activity [Guy, *et al.*, 1994]. The stimulation of tyrosine phosphorylation of neu by NRG might suggest direct activation of neu by NRG, but in view of previously published work, is more likely to reflect interaction with endogenous erbB-3. However, erbB-4 is able to bind and respond to NRG directly.

NRG induces extensive cross-talk among receptors expressed in binary combinations (Figure 1). Either erbB-3 or erbB-4, both of which bind NRG, enable regulation of the EGFR by NRG. This is the first evidence that NRG can regulate EGFR signaling. As predicted from earlier work, NRG stimulates tyrosine phosphorylation of both receptors in the neu + erbB3, neu + erbB-4, and erbB-3 + erbB-4 cell lines. Coexpression of EGFR, neu, or erbB-4 with erbB-3 permits NRG induction of erbB-3 tyrosine phosphorylation. Although earlier work showed that expression of neu enhances tyrosine phosphorylation and NRG regulation of erbB-3 [Carraway, *et al.*, 1994], that work was done in COS-7 cells, which express significant basal amounts of neu and EGFR. Thus the present work demonstrates for the first time that *de novo* expression of either the EGFR, neu, or of erbB-4 enables hormone-regulated phosphorylation of erbB-3. Endogenous erbB receptor expression in Ba/F3 cells played a limited, yet significant role in specifying responses

to NRG stimulation in these experiments. While endogenous erbB-3 expression permits NRG stimulation of exogenous neu tyrosine phosphorylation, NRG does not stimulate receptor tyrosine phosphorylation in cells that express exogenous EGFR only. Perhaps the level of endogenous erbB-3 expression in Ba/F3 cells is insufficient to permit NRG-induced EGFR tyrosine phosphorylation. Alternatively, intrinsic differences between the exogenous human and endogenous mouse proteins may result in the differing capacities to undergo NRG-induced heterotypic receptor interactions.

While NRG can stimulate the tyrosine phosphorylation of each receptor under the appropriate conditions, the diversity of biological responses to NRG indicates that there must be additional mechanisms by which biological responses to NRG are specified. The patterns of NRG-induced stimulation of erbB receptor tyrosine phosphorylation and IL-3 independent survival or proliferation demonstrates that there are several hierarchical levels at which biological responses to NRG are apparently specified.

First, responsiveness to NRG requires the expression of erbB-3 or erbB-4. Previous work and results presented here establish that NRG can not bind or stimulate tyrosine phosphorylation of erbB family receptors in the absence of erbB-3 or erbB-4 expression. Biological responses to NRG are also specified by the intrinsic kinase activity of the erbB family receptor(s) stimulated by NRG, since the kinase-deficient erbB-3 requires the presence of a co-receptor for hormone-regulated phosphorylation.

Distinct biological responses to NRG are also conferred by ligand-induced coupling of different erbB family receptors to different signaling pathways. NRG enables the IL-3 independent survival of cell lines expressing neu, probably through NRG-induced activation of neu via erbB-3. However, NRG does not enable the IL-3

independent survival of EGFR + erbB-3 cells, or of erbB-4 cells, even though NRG stimulates receptor tyrosine phosphorylation in these lines. This demonstrates that neu has signaling properties distinct from those of the EGFR, erbB-3, or erbB-4, and is consistent with earlier work showing that different erbB family receptors can activate different signaling pathways and responses [DiFiore, *et al*, 1990; Fedi, *et al.*, 1994; Kim, *et al.*, 1994; Prigent and Gullick, *et al.*, 1994; Soltoff, *et al.*, 1994; Carraway, *et al.*, 1995]. Neither NRG nor EGF induces IL-3-independent proliferation of cells that individually express erbB-4 or EGFR [Riese, *et al.*, 1995; Riese, *et al.*, 1996]. Yet, NRG stimulates IL-3-independent proliferation in the EGFR + erbB-4 cell line. One simple explanation would be that IL-3-independent proliferation requires activation of two independent pathways, one of which is activated by the EGFR, and one by erbB-4. An interesting alternative would be that EGFR and erbB-4 phosphorylation sites differ in ligand-induced EGFR/erbB-4 heterodimers than in ligand-induced receptor homodimers owing to substrate specificity of the receptor catalytic domains and steric considerations in the cross-phosphorylation reaction. This would permit recruitment of unique signaling proteins to the heterodimer, resulting in unique biological responses. Thus, the diversity of hormone-regulated outputs from this receptor outwork may extend beyond the simple combinatorial possibilities.

Since EGF and NRG bind to different receptors, it can be predicted that individual members of the EGF family of ligands activate different constellations of erbB family receptors, so that these different ligands will yield distinct patterns of biological responses. Evidence presented here supports this prediction. Previous reports demonstrated that betacellulin binds to the A431 human adenocarcinoma cell line and the MDA-MB-453 human breast carcinoma cell line, both of which overexpress the EGFR. This binding was quenched by the addition of an excess of EGF, suggesting that betacellulin is a ligand for the EGFR [Watanabe, *et al.*, 1994].

However, the effect of betacellulin on EGFR tyrosine phosphorylation and signaling and the possibility that betacellulin might activate other erbB family receptors were not assessed. Here we show that in Ba/F3 cells expressing only a single ectopic erbB family receptor, betacellulin stimulates the tyrosine phosphorylation of both the EGFR and, surprisingly, erbB-4 (Figure 1). This is consistent with the observation that radiolabeled betacellulin binds specifically to EGFR and erbB-4, but not to neu (Plowman, *et al.*, in preparation). Control experiments performed in parallel demonstrated that radiolabeled amphiregulin and EGF bound only to EGFR and radiolabeled NRG-β bound only to erbB-4, as previously reported. Thus, betacellulin exhibits activities that are distinct from those displayed by EGF, which activates the EGFR alone, and NRG-β, which activates erbB-3 and erbB-4 (Figure 1). Furthermore, in this first comprehensive analysis of erbB family transmodulation for both betacellulin and EGF, we find that EGF can transmodulate erbB-4 in the EGFR + erbB-4 cell line (Figure 1). We also demonstrate that betacellulin stimulates a pattern of receptor transmodulation that is qualitatively distinct from the patterns stimulated by EGF and NRG-β.

With one exception, betacellulin, EGF, and NRG-β transmodulated the tyrosine phosphorylation of all four erbB family receptors in cell lines that express any receptor for each ligand (Figure 1). Thus, differences in ligand activities in the double recombinant cell lines can be predicted by differences in activities in the single recombinant cell lines. For example, NRG-β activates erbB-3, while betacellulin does not activate neu or erbB-3. Not surprisingly, in cells expressing neu + erbB-3, NRG-β stimulates the phosphorylation of both receptors, while betacellulin does not stimulate the phosphorylation of either receptor in this cell line (Table 1). The single exception is the response of the erbB-3 + erbB-4 (3+4) cell line to betacellulin. Both betacellulin and NRG-β stimulate erbB-4 tyrosine

phosphorylation in the single recombinant cell line. However, in 3+4 cells, NRG-β stimulates the tyrosine phosphorylation of both receptors, while betacellulin stimulates the tyrosine phosphorylation of erbB-4 but not of erbB-3 (Figure 1). Nonetheless, because NRG-β binds erbB-3, it is not clear that this absence of erbB-3 tyrosine phosphorylation is due to differences between betacellulin- or NRG-β-induced erbB-3 transmodulation.

Previous work demonstrated that different erbB family receptors or combinations of receptors couple to distinct cellular signaling pathways. For example, EGFR activation stimulates the tyrosine phosphorylation of four proteins that are not highly phosphorylated following neu activation [Fazioli, *et al.*, 1992]. Furthermore, activated erbB-3 stimulated higher levels of phosphatidylinositol 3-kinase than EGFR did [Fedi, *et al.*, 1994; Soltoff, *et al.*, 1994; Carraway, *et al.*, 1995], and it has been suggested that EGFR and neu bind the adapter protein GRB2, but erbB-3 does not [Prigent and Gullick, 1994; but also see Kim, *et al.*, 1994; Fedi, *et al.*, 1994]. These different coupling capacities of the erbB family receptors can be correlated to specific biological responses. Activation of the EGFR stimulates the IL-3 independent proliferation of 32D myeloid cells, while wild-type and mutationally-activated neu alleles do not [DiFiore, *et al.*, 1990]. In Ba/F3 cells, however, activation of neu stimulates IL-3 independent survival, while activation of EGFR and erbB-4 together stimulates IL-3 independent proliferation [Riese, *et al.*, 1995; Riese, *et al.*, 1996].

We found that betacellulin stimulates IL-3 independent survival or proliferation in neu + erbB-4 cells and in every cell line that expresses EGFR. In contrast, EGF stimulated IL-3 independence only in those cell lines that express EGFR while NRG-β stimulated IL-3 independence only in the EGFR + erbB-4 cell line and in those cell lines that express neu [Riese, *et al.*, 1995; Riese, *et al.*, 1996].

Therefore, with a single exception, the minimal requirement for IL-3 independence is activation of either EGFR or neu. The exception is that betacellulin and EGF, but not NRG-β, stimulated IL-3 independent survival in the EGFR + erbB-3 cell line (Table 1). This lack of response to NRG-β may merely reflect the lower level of EGFR phosphorylation stimulated by NRG-β in this cell line [Riese, *et al.*, 1995]. On the other hand, the absence of biological response may reflect the different mechanism by which the EGFR is activated. We demonstrated previously that coupling of these multiple receptor species to cellular signaling pathways acts in a non-additive manner in specifying biological responses [Riese, *et al.*, 1996]. Accordingly, while activation of EGFR or neu by themselves stimulated IL-3 independent survival, activation of EGFR and either neu or erbB-4 together stimulated IL-3 independent proliferation (Table 1).

As we have discussed previously, biological responses to EGF family ligands are regulated by several hierarchical mechanisms. Some, but not all, of these mechanisms are shared by other networks of receptor tyrosine kinases and their ligands, including the neurotrophin network and the fibroblast growth factor (FGF) network. Like the EGF family, the neurotrophin and FGF ligand families have several members that can each activate multiple receptors. The neurotrophin ligand family includes nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4, also known as NT-4/5 or NT-5 [Reviewed in Barbacid, 1994], while the FGF family has at least 9 members encoded by different genes [Reviewed in Johnson and Williams, 1994]. Furthermore, like the erbB receptor family, both the FGF receptor and neurotrophin receptor families have multiple members (FGFR-1, FGFR-1, FGFR-3, FGFR-4 and TrkA, TrkB, TrkC, respectively). Moreover, like the EGFR and erbB-4, some of these FGFRs and Trks can bind multiple ligands [Reviewed in Johnson and Williams, 1994; Barbacid, 1994].

Another regulatory mechanism common to the EGF/erbB and FGF signaling networks is that both use heparan sulfate proteoglycans (HSPGs) to modulate receptor-ligand interactions. FGFs bind with low affinity in a multivalent manner to HSPGs, causing ligand oligomerization [Reviewed in Lemmon and Schlessinger, 1994] and increasing their binding affinity for FGFRs [Reviewed in Eckenstein, 1994]. Because the FGF/FGFR complex exists in a 1:1 stoichiometry [Spivak-Kroizman, *et al.*, 1994], yet FGFs are monomeric, it has been proposed that HSPG binding potentiates FGF stimulation of FGFR phosphorylation and dimerization. HSPGs also regulate the interactions of EGF family ligands with their receptors. Several EGF family ligands bind HSPGs, including NRGs, amphiregulin (AR), and heparin-binding-EGF-like growth factor (HB-EGF), and this binding regulates ligand-receptor interactions [Aviezer and Yayon, 1994; Johnson and Wong, 1994; Cook, *et al.*, 1995a; Cook, *et al.*, 1995b]. However, many of the mechanistic details of regulation by HSPGs have yet to be elucidated.

While the neurotrophin and FGF networks have regulatory mechanisms that are also features of the EGF/erbB network, there are also features of the neurotrophin and FGF networks that are not part of the EGF/erbB network. Alternative splicing produces truncated FGFR and Trk isoforms lacking the cytoplasmic tyrosine kinase domain and sites for tyrosine phosphorylation [Reviewed in Johnson and Williams, 1994; Barbacid, 1994. Therefore, a regulatory mechanism not observed in the EGF/erbB network results in dominant negative receptors, which are not a characteristic of the EGF/erbB network. Another feature that is characteristic of the neurotrophin network and not seen in the EGF/erbB network is regulation by a low-affinity co-receptor. p75, the low-affinity neurotrophin receptor, has no tyrosine kinase domain [Reviewed in Chao, 1994] and p75 binding is in some cases dispensable for biological response [Reviewed in Ibanez,

1994]. Nonetheless, it has been proposed that p75 regulates the biological response to neurotrophins by altering the affinity of neurotrophin binding to the Trk family receptors [Benedetti, *et al.*, 1993; Reviewed in Chao, 1994].

Data presented here suggests that differences in NRG-β, EGF, and betacellulin activities play a significant role in specifying the proliferation and differentiation of human tissues *in vivo*. These ligands may play their most significant role in the mammary epithelium and tissues of neuroectodermal origin. Not only has the expression of EGF family ligands been documented in these cell types, but these ligands can regulate the proliferation and/or differentiation of these cell types in cultured cell or animal model systems. Furthermore, mounting evidence suggests that, depending on the identity of the receptors, increased expression and/or signaling of erbB family receptors may either stimulate or inhibit epithelial cell proliferation and tumorigenesis. [Reviewed in Hynes and Stern, 1994; Bacus, *et al.*, 1996]. Because different erbB family receptors stimulate different physiological responses through differential coupling to cellular signaling proteins, a major focus of our current efforts has been to identify the components of these signaling pathways. These experiments may identify genes suitable for anti-tumor gene therapy or proteins that are targets for anti-tumor small molecule inhibitors.

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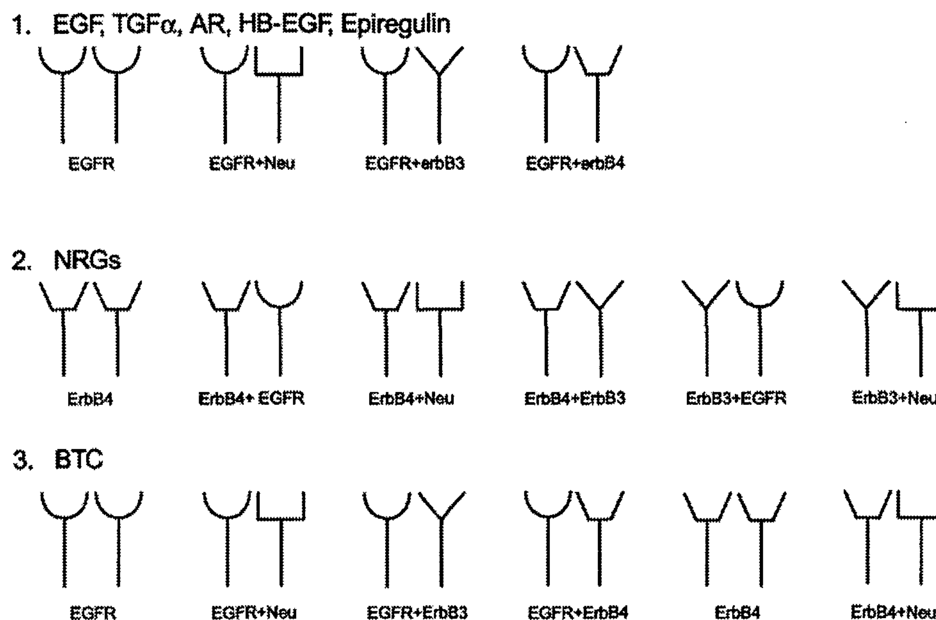
VI. Appendices

A. Figures and Tables

Figure 1

Patterns of Hormone-induced ErbB Family Receptor Phosphorylation



Data adapted from Riese, *et al.*, 1995; Riese, *et al.*, 1996; Riese, *et al.*, in press.

Table 1

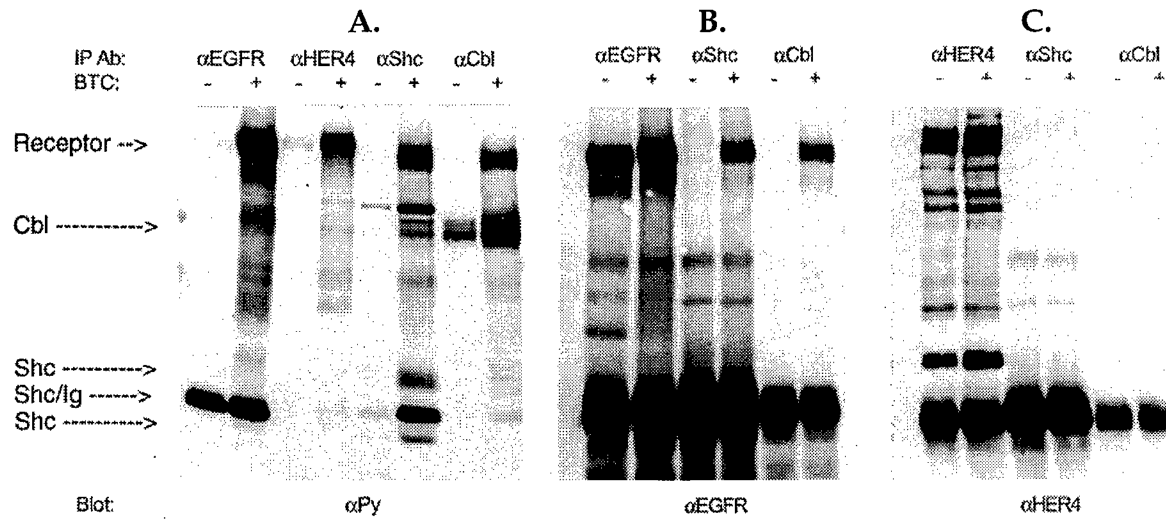
Physiologic Responses to ErbB Family Receptor Activation

Receptor(s)	Response
EGFR	IL-3-Independent Survival
Neu	IL-3-Independent Survival
ErbB-4	No Response
EGFR + Neu	IL-3-Independent Proliferation
EGFR + ErbB-4	IL-3-Independent Proliferation
Neu + ErbB-4	IL-3-Independent Survival

Data adapted from Riese, *et al.*, 1995; Riese, *et al.*, 1996; Riese, *et al.*, in press.

Figure 2

Immunoprecipitation and Immunoblotting of Lysates From EGFR+ErbB4 Cells Stimulated with Betacellulin



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D. Graduate degrees resulting from contract support:
None