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PRINCIPAL INVESTIGATOR: David F. Stern, Ph.D.

CONTRACTING ORGANIZATION: Yale University
New Haven, CT 06520-8047

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David F. Stern, Ph.D.

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The gene encoding the neu/erbB-2/HER-2 receptor tyrosine kinase is one of a small number of genes known to be altered in human breast and ovarian carcinoma. It is amplified and/or over expressed in approximately one-fourth of these cancers. With its probable role in etiology, and expression at the cell surface, neu has great potential as a prognostic indicator and therapeutic target. Efforts to exploit this receptor in the clinical arena have been impeded by the failure to consider regulation of neu by hormones and co-receptors. This has been complicated by the fact that at least seven different peptide hormones, epidermal growth factor (EGF), transforming growth factor-alpha, amphiregulin, betacellulin, heparin-binding EGF-like growth factor, epiregulin, and the diverse neuregulins are all able to activate neu. They work by binding to the co-receptors EGF receptor, erbB-3 and erB-4, which then dimerize with neu. Work by this laboratory has shown that each one of these factors activates a different constellation of receptors, leading to a great diversity of possible response. Moreover, the coupling of cellular responses to each factor is determined by the subset of receptors expressed in each tissue. Thus in order to interpret the function of any one of the factors or receptors, including neu, it is essential to consider the influences provided by the entire set.

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INTRODUCTION

Breast cancer is one of the most important cancers afflicting women and presents challenging treatment decisions. After p53 alterations, the most frequent change in an identified gene is amplification and/or overexpression of the *neu/erbB*-2/*HER*-2 gene, which occurs in up to one-third of breast cancers. The gene product (denoted p185 or non-italicized neu), is a receptor tyrosine kinase (RTK) [1, 2, 3]. This gene was originally discovered in mutant form in chemically-induced rat nervous system tumors and is now known to be a member of the Type 1, or epidermal growth factor (EGF) receptor gene (*erbB*) family. The family includes four receptors, which will be referred towill be referred to herein as the EGFR, neu, erbB-3, and erbB-4. (See our review, [4], Appendix). Small-scale screens for *neu* alterations in human tumors led to discovery of changes in a number of adenocarcinomas including breast [5], ovarian, gastric, bladder, lung, and colon. Two influential studies showed that *neu*, and not a panel of other oncogenes is amplified in breast and ovarian carcinomas and that this amplification correlates with RNA and p185 overexpression [6, 7]. Numerous studies of *neu* in mammary carcinoma have led to the following conclusions [4]:

1.*neu* is amplified in 20-30% of mammary carcinomas, with the frequency of amplification higher in tumors from patients with affected lymph nodes [8, 9, 10, 11, 12, 13, 14, 15, 16]. The gene amplification suggests that there is a selection in the tumors for *neu* overexpression. (It cannot be absolutely ruled out that neighboring genes are selected for amplification.)

2.Amplification correlates well with concomitant RNA and p185 expression [6, 7]. An additional 5% of specimens overexpress the receptor without obvious changes in gene structure or copy number [5, 11, 15, 17].

3.There is no evidence for structural mutations in p185 in human tumors. This negative result is weak owing to the high copy number of genes and the large size of the

mRNA. Recent work in the mouse transgenic system suggests that this issue should be reconsidered [18].

4.Amplification and/or p185 overexpression can be found in all grades and stages of carcinomas, but not hyperplasia or dysplasia. It is found more frequently in ductal carcinoma in situ (DCIS) than in infiltrating ductal carcinoma (IDC)[14, 16, 19, 20].

5.Amplification and/or p185 overexpression is associated with poor prognosis, especially in node-positive patients. However the extent of this association and independence from other prognostic markers varies greatly among different studies (reviewed, [4]).

Taken together, these data indicate that *neu* amplification and overexpression play a major role in mammary carcinogenesis. This is consistent with findings in model systems: i) the mutated rat *neu* oncogene is as potent as any including *ras* in tissue culture systems. ii)In contrast to other growth factor receptors including the EGFR, overexpression of p185 in the absence of ligand is sufficient to transform cells [21, 22]. iii)Transgenic mice harboring a mutationally activated *neu* oncogene develop multi-focal mammary carcinoma when expressed under control of a murine mammary tumor virus (MMTV) promoter, which confers high level expression in mammary gland and a few other tissues (not found in all studies [23, 24]. iv)Perhaps most compelling is the fact that transgenic mice carrying a structurally normal *neu* gene driven by the MMTV promoter develop metastatic mammary carcinomas [25]. This is noteworthy because it reconstructs what appears to be occurring in human cancer: overexpression of normal p185 in mammary tissue.

Since p185^{neu} is a cell surface protein that seems to play a causal role in mammary carcinogenesis, it is under intensive investigation as a therapeutic target [4, 26]. Phase II clinical trials, in which patients were infused with anti-neu antibody 4D5 have been completed with a roughly 15% response rate, and represent the vanguard for expanded therapeutic trials targetting this receptor (J. Baselga, personal communication). In spite of

the findings linking *neu* to mammary carcinoma, and despite the fact that patients are already being exposed to neu antagonists, little is known about the function of neu either in the organism, or in breast cancer.

The physiological function of p185*neu*, like any hormone receptor, can only be understood in the context of the hormones that regulate it. The EGFR is activated by binding of at least six different peptide hormones, EGF, TGF-α, amphiregulin (AR), betacellulin (βC), epiregulin [27](epi) and heparin-binding EGF-like growth factor (Hb-EGF)[28, 29, 30, 31]. p185, by itself, cannot bind or be activated by these hormones (epi has not been tested). We discovered that EGF and TGF-α, which do not bind to p185, activate p185 Tyr phosphorylation and stimulate p185-associated kinase activity [2, 32]. This phenomenon, now termed **transmodulation**, is dependent upon the co-expression of the EGF receptor with p185 [33, 34, 35, 36]. It probably occurs at least in part through formation of receptor heterodimers [37, 38]. Transmodulation of neu by the EGFR is biologically relevant since it works with EGF, TGF-α, betacellulin (see below) and AR, stoichiometrically activates p185 [32], permits association of substrates [39], and correlates with *in vivo* synergy in transforming ability of these two receptors [40]. Thus wherever the two receptors are co-expressed, EGFr agonists activate *neu* . In cell lines that express both receptors, EGF-regulated neu signaling is at least as important as signaling by the EGFR [41]. Since p185 and the EGFR have distinguishable signaling activities [42], this means that regulation of neu production provides a means to alter the signal coupled to EGF.

The transmodulation of neu by the EGFR is a prototype for other interactions within the Type 1 receptor family discovered more recently. Ignorance of these interactions has confused many groups studying the EGFR and resulted in a rather muddy literature which is just now being rationalized [4, 43]. For example, several laboratories independently identified an activity termed Heregulin, neu diffentiation factor, gp30, p75, neuregulin, ARIA, and Glial Cell Growth Factor (GGF) [44, 45, 46, 47, 48, 49, 50, 51], a family of related proteins evidently produced by alternate splicing[50](They

will be referred to collectively here as NRG, for the composite name neuregulin, or as NDF). At first the NRGs seemed to be neu ligands since they activate p185 tyrosine phosphorylation in the absence of the EGFR and could be cross-linked to neu [45, 49]. However, it is now known that NRGs bind to both erbB-3 and erbB-4 which can then activate neu by transmodulation [52, 53]. A further complication of this receptor system is that erbB-3 lacks robust kinase activity, and itself requires a second receptor for activity [54].

Additional candidates for neu ligands have been identified but not yet expressed in recombinant form and tested for activity [55, 56]. Nonetheless, the independent purification of NRGs by three different laboratories seeking the *neu* ligand suggests that in mammary epithelia the significant inputs to *neu* may come through transmodulation: transmodulating agonists TGF-α, AR, and NDFs are often produced in mammary tissue or cell lines [57, 58, 59] as are the cooperating receptors. Even if these hormones are uniquely responsible for neu activation in mammary tissue, the biological complexities may be enormous. TGF-α and AR, although both EGFR agonists, have somewhat different biological activities [28]. NRGs at first seemed to have radically different activities than EGF agonists since they promote differentiation in some cell lines [49, 60] (but not others [45, 50]), but this has still not been verified in tissue[4].

In summary, *neu* amplification and overexpression is likely to play a significant role in carcinomas where it occurs. However, the presence of activating mutations and agonistic peptide hormones will regulate *neu* function much more strongly than abundance. The Type 1 RTKs comprise a network in which the signaling potential of each receptor is conditioned not only by the presence of hormones, but is further regulated by the co-expression of related RTKs. The long-term focus of this grant is to define the capabilities of the erbBfamily receptor network: the spectrum of hormones that activate each receptor and receptor combination, and the differences among signalling pathways governed by these receptor systems. These objectives include:

Aim 1: Signalling of individual receptors and receptor combinations will be compared by investigating receptor phosphoryaltions and substrate phosphorylations to determine how receptor interactions modulate signalling specificity.

Aim 2: Functions of NRG ecto- and endo-domains will be analyzed.

Aim 3: Biological activity of NRGs and NRG/TGF-α combinations will be determined in tissue culture.

BODY

ErbB Receptor Signaling Network

The foremost objective for year 1 (Tasks 1a and 1c) was to determine the ability of each receptor and receptor combination to respond to each of the EGF family agonists. This was done by using stable cell lines produced in year 1 that express erbB, erbB-2, erbB-3, and erbB-4 singly and in all pairwise combinations. In year 1 we completed most of the analysis of the ligands EGF, TGF-α, AR, Betacellulin, NRG, and HbEGF [61]. This work was completed in year 2, with publication of 2 additional papers [62, 63], included in the Appendix. In year 2 we undertook analysis of the remaining EGF-related factor described in the original proposal, Cripto-1. Since the goal of this work is a comprehensive analysis of EGF-related factors, we also felt obligated to investigate two factors discovered more recently than the original proposal: epiregulin [27], and Cerebellar-Derived Growth Factor (CDGF)[64]. This work is still in progress, but thus far it appears that Cripto-1 is unable to activate any of the receptors, even though it can activate erbB-4 in a mammary carcinoma cell line, that CDGF is functionally similar to its relative NRG, and that epiregulin may be unique in requiring two receptors for a response. A manuscript describing the CDGF work has already been submitted for publication [64]. This work should be complete by mid-year 3, which will mark the final completion of Task 1a and 1c unless more factors are discovered.

Task 1b was to survey substrate phosphorylations in activated Ba/F3 cells. This has been done using phospho-tyrosine immunoblots to identify growth factor-regulated phospho-proteins. The spectrum of phosphorylated substrates correlated with the particular receptor activated, rather than the particular growth factor used. Since the survival and proliferation assays suggest that EGFR and erbB-4 (activation of both was required for proliferation) perform different functions, we have been especially interested in differences between EGFR and erbB-4 substrates.

In Ba/F3 cells, EGFR activation was accompanied by the phosphorylation of c-Cbl and Shc, two known signaling effectors for EGFR. In contrast, activated erbB-4 was not accompanied by c-Cbl or Shc phosphorylation. 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 stimulated high levels of EGFR, erbB-4, Shc, and c-Cbl phosphorylation. However, Shc and c-Cbl preferentially complexed with EGFR and not with erbB-4, suggesting again that Shc and c-Cbl are signaling effectors for EGFR and not for erbB-4. 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.

Aim 1, Task 2. Mammary Cell Lines. There is mounting evidence that while activation of either EGFR or erbB-2 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. 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 or BTC. Therefore, we tried to establish MCF-10A derivatives that ectopically overexpress erbB4 through infection with a recombinant retrovirus containing the neomycin resistance gene and the human erbB4 cDNA . We noted that this retrovirus stock had an unusually 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. Retrovirus stocks harboring erbB-4 showed a much higher ratio of fibroblast/MCF10 colony-forming units than did control retroviruses, and the few infectants recovered failed to express high levels of erbB-4. This

suggests that erbB-4 is indeed a growth inhibitor of MCF-10A cells, probably through induction of differentiation.

II.NRG Intracellular Domain

The cytoplasmic domain of NRG family EGF-related growth factors is unusual in showing extraordinary diversity of regulation by splicing, and in that some forms have unusually long cytoplasmic tails of unknown function (over 400 amino acids). We hypothesize that these tails are likely to themselves transmit signals so that binding of NRG ecto-domains to the cognate receptors results in bidirectional signaling. As a first step in addressing this problem, we have begun to clone and express NRGs in transient and stable transfected cell lines.

Tasks 3a and 3b. Using transient expression of NRG in Cos-7 cells, and stable expression in NIH3T3 cells, we have found that cell surface expression of NRG induces a stable and tight binding of Ba/F3 cells expressing erbB-4 or erbB-2 and erbB-4 to the NRG-expressing cells. Thus membrane anchored NRG may indeed serve as an adhesion receptor. To test our hypothesis that NRG induces a cell-autonomous signal when activated, we have analyzed the phenotype of stable cell lines expressing full-length or truncated NRG. It appears that the presence of the cytoplasmic tail is associated with growth inhibition, although this provisional result remains to be quantitatively verified. If it is, this important finding would for the first time suggest a physiological function for the NRG cytoplasmic domain. In a second approach, we have produced and successfully expressed a GST- NRG tail fusion protein in bacteria that will be used for affinity purification of binding proteins. Finally, yeast strains are ready to conduct a two-hybrids screen for proteins binding to segments of the NRG tail. All of this work (Task 3b) should proceed rapidly over year 3.

Task 4, Mammary Fat Pad Implantation Experiments.

We rearranged our original schedule by moving the mammary tissue pellet implant experiments up to years 1 and 2. Pellets were impregnated with NRG or TGF-α without or with estrogen/progesterone. Implants were inserted into the the surgically-exposed number 4 mammary fat pad of 32 da Balb/c females, with growth factor-free pellets used as controls in the contralateral mammary fat pad. Four days after implantation whole mounts were prepared and stained with hematoxylin.We favor this approach because the epithelium responds in the context of normal matrix and hormonal environment and hence is highly physiological, and has the collateral advantages of being fast, manipulable and inexpensive. Although there is considerable literature for both EGF and TGF-α, the NRGs have not been investigated in this format. These experiments are now complete and have resulted in a publication [65] . There were two very exciting results from this work. First, we found that although NRGs and TGF-α can both enhance ductal extension and branching and lobuloalveolar development, only lobuloalveoli induced by NRG α or β contained secretory products. Thus only the NRGs can induce full terminal differentiation. This is consistent with the finding that TGF-α is expressed throughout postnatal mammary development, but NRG is only expressed in pregnancy. A second exciting result was that NRG-α was more potent than NRG-β in these assays. This is a preliminary result owing to the difficulty in ensuring that biological activities of the preparations were matched, but is intriguing: Birchmaier has reported that NRGα, but not NRGβ, is expressed in mammary tissue. On the other hand, in all reports comparing activity of these hormones, they are qualitatively similar, but **NRG**β is more potent. Hence our new results suggest for the first time a mammary-specific preference of unknown mechanism for responsiveness to NRGα, perhaps even involving an accessory receptor.

The alternative method for implanting factors will be to present cell surface-anchored hormones on Balb/c 3T3 cells. Production of the cell lines (Task 4a) is nearly

complete, a few months behind schedule, and the implantations will probably be completed a year ahead of schedule by the end of year 3.

CONCLUSIONS

This has been a transition year, in which we have finished much of the work initiated in year 1, and laid the groundwork for new frontiers in year 3. In addition to a publication in year 1[61], we have published three new papers [62, 63, 65] this year. and submitted a fourth [64]. We have completed a major fraction of the original proposed work, and have been faithful to the original timetable. The definition of the abilities of individual EGF-related hormones to regulate specific receptors and receptor combinations is absolutely essential for understanding the biology of any of these hormones and their receptors and is a significant contribution of this work. This "wiring diagram" will benefit all investigators working in the system and is a unique contribution of our laboratory. The discovery of new EGF-related hormones makes this goal a moving target, but we will continue to fill out this elemental work.

This year has seen important strides in our understanding of the different phsyiological functions of TGF-α and NRGs in mammary tissue. Thus for the first time we can definitively identify a truly physiological differentiative response with NRGs. Moreover, the possibility that mammary tissue has a priveleged response to NRGα over NRGβ raises many interresting possibilities.

We have also made progress, albeit slowly, on identifying a function for the NRG cytoplasmic domain. It appears to inhibit growth of cells, either by inducing apoptosis, or by inhibiting proliferation. The coming year should produce a much more concrete description of cellular responses to the tail. Finally, we have now completed the groundwork for doing 2-hybrids and affinity purification screens to identify tail-associated proteins.

The difficulties in engineering MCF-10A cells to express erbB-4 may impede some of the original experiments proposed for task 2c, but answers a major biological question that these experiments were to address, namely the differences in cellular responses coupled to each of the receptors in mammary backgrounds. If we find, as we suspect, that

the growth inhibition is due to differentiation, the work will be completely consistent with the implant experiments, and will provide strong evidence that the NRG/erbB-4 couple is a major regulator of mammary differentiation. This has implications for breast cancer patients, since active erbB-4 would be associated with a favorable prognosis, and may suggest hormone therapies based on NRG, or other erbB-4 activators.

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APPENDIX
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The Cellular Response to Neuregulins Is Governed by Complex Interactions of the erbB Receptor Family

DAVID J. RIESE II,[1] TOM M. VAN RAAIJ,[1] GREGORY D. PLOWMAN,[2]† GLENN C. ANDREWS,[3] AND DAVID F. STERN[1]*

Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06520-8023[1]; Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Washington 98121[2]; and Pfizer Central Research, Groton, Connecticut 06340[3]

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Deregulated signaling by the four members of the epidermal growth factor receptor tyrosine kinase family (erbB family) is implicated in the genesis or progression of human cancers. However, efforts to analyze signaling by these receptors have been hampered by the diversity of ligands and extensive interreceptor cross talk. We have expressed the four human erbB family receptors, singly and in pairwise combinations, in a pro-B-lymphocyte cell line (Ba/F3) and investigated the range of interactions activated by the epidermal growth factor homology domain of the agonist neuregulin β. The results provide the first comprehensive analysis of the response of this receptor family to a single peptide agonist. This peptide induced complex patterns of receptor tyrosine phosphorylation and regulation of Ba/F3 cell survival and proliferation. These data demonstrate the existence of several previously undocumented receptor interactions driven by neuregulin.

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) is implicated in human mammary cancer, ovarian cancer, gastric cancer, and glioblastoma (reviewed in reference 19). Understanding the normal and pathological functions of these receptors requires that their regulation by hormones be elucidated. One complication is that there are at least 15 different agonists for erbB family receptors, including EGF, transforming growth factor α, amphiregulin, betacellulin, heparin-binding EGF-like growth factor, and the several differentially spliced variants of the neuregulins (NRGs), also known as gp30 (27), heregulins (18), neu differentiation factors (35, 54), glial growth factors (28), and acetylcholine receptor-inducing activity (5, 12). Some of these factors bind to and activate signaling by more than one receptor. Moreover, these ligands stimulate nonadditive receptor interactions in cells expressing multiple erbB receptor family members. For example, EGF activates neu when coexpressed with the EGFR, but EGF does not bind or activate neu expressed on its own (22, 50). This transmodulation activation of neu by EGFR apparently works through the formation of EGF-driven receptor heterodimers (15, 53).

NRGs were initially identified as candidate neu ligands by their ability to induce neu tyrosine phosphorylation. The longest forms of NRG contain several different modular domains, including a kringle fold, a C-2 immunoglobulin-like domain, a putative heparan sulfate proteoglycan attachment site, sites for N- and O-linked glycosylation, an EGF homology domain, a hydrophobic membrane-spanning domain, and an intracellular domain of variable length (6, 18, 28, 35, 54). Tissue-specific alternative splicing of NRG transcripts from a single gene results in many NRG isoforms containing different sets of these motifs. Moreover, alternative splicing also produces two types of EGF domain, designated α and β (55). α and β isoforms have different biological activities, which may in part reflect their differential binding affinities to cells expressing receptors for NRG (26).

NRGs are likely to play a significant role in regulating cellular proliferation and differentiation in vivo. NRGs were initially purified from medium conditioned by *ras*-transformed Rat-1 fibroblasts (35) or by the MDA-MB-231 human mammary tumor cell line (18), suggesting that NRGs establish or maintain the growth-transformed phenotype. NRG also affects the proliferation and differentiation of cultured mammary cells. NRG stimulates (18) or inhibits (35, 54) the in vitro proliferation of human mammary tumor cells, which frequently overexpress erbB family receptors (reviewed in reference 19), while NRG stimulates proliferation and milk protein synthesis in a cultured mouse mammary epithelial cell line (29). NRG may also promote wound healing. A single NRG isoform accelerates epidermal migration via increased terminal differentiation of epidermal cells and stimulates integrin expression in the epidermis during wound healing, while wounding stimulates NRG expression in dermal fibroblasts adjacent to the wound (9). NRG also modulates the differentiation and proliferation of neuroectodermal cells. NRGs act as glial cell growth factors (28), may specify a glial cell fate for neural crest stem cells (45), appear to mediate axon-induced mitogenesis of Schwann cells (32), and stimulate acetylcholine receptor synthesis at neuromuscular junctions (5, 12, 20). Furthermore, NRG expression patterns suggest important functions in neurogenesis and in mesenchymal-epithelial cell interactions during development (6, 28, 30, 33).

The physiological responses to agonists for erbB family receptors depend 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 (7, 36, 39, 48, 51). Instead, NRG binds erbB-3 (2, 23, 48, 51) and erbB-4 (7, 38, 39, 51). Coexpression of erbB-3 or erbB-4 with neu permits NRG-induced tyrosine

* Corresponding author. Mailing address: Department of Pathology, Yale University School of Medicine, P.O. Box 208023, New Haven, CT 06520-8023. Phone: (203) 785-4832. Fax: (203) 785-7467. Electronic mail address: STERN@BIOMED.MED.YALE.EDU.

† Present address: Sugen, Inc., Redwood City, CA 94063-4720.

phosphorylation of neu, presumably through the formation of neu–erbB-3 or neu–erbB-4 heterodimers (2, 23, 39, 48). Despite the many combinatorial possibilities afforded by assortment of four different receptors, interactions of erbB family members with their agonists have been investigated only 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, including NRGs.

To address these issues, we have undertaken a parallel analysis of the aggregate signaling potential of this receptor family by expressing all four human erbB family receptors, singly and in each pairwise combination, in a uniform cell background. We have used the resulting cell lines for the first comprehensive evaluation of NRG-induced erbB family receptor activation and coupling to cellular responses. The results reveal the pattern of activation of these receptors by NRG and identify novel ligand-induced interactions among these receptors. Moreover, these data suggest several distinct mechanisms by which biological responses are specified by interactions among erbB family receptors and their agonists.

MATERIALS AND METHODS

Cell lines and cell culture. The Ba/F3 mouse pro-B-lymphocyte cell line (34) 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 (8). Ba/F3 derivatives transformed with constructs expressing erbB family receptors were grown in medium supplemented with 200 μg of G418 (Gibco/BRL) per ml.

Plasmid constructions. The *Sac*II-*Xho*I fragment of pCO12EGFR (52), which contains the full-length human EGFR cDNA, was subcloned into the *Sma*I site of pBluescript SK−, generating pSKEGFR. The EGFR expression vector pLXSN-EGFR used in the experiments described here was constructed by cloning the 4.2-kb *Xho*I fragment of pSKEGFR, which contains the complete human EGFR cDNA, into the *Xho*I site of the recombinant retroviral expression vector plasmid pLXSN, which carries a neomycin resistance gene under the transcriptional control of the simian virus 40 early promoter (31). The neu expression vector pLXSN-Long-Neu was constructed by cloning the 4.8-kb *Nru*I-to-*Dra*I fragment of pCDNEU (39), which contains the complete human neu cDNA as well as 714 bp of vector sequences 5′ to the neu transcriptional start site, into the *Hpa*I site of pLXSN. Subsequently, the vector sequences 5′ to the neu transcriptional start site were removed by cloning a 4.1-kb *Xho*I fragment of pLXSN-Long-Neu into the *Xho*I site of pLXSN, generating the neu expression vector pLXSN-Neu used in these studies. The erbB-3 expression vector pLXSN-erbB-3 was constructed by cloning the 4.3-kb *Bss*HII fragment of pBSHER3X (40), which contains the complete human erbB-3 cDNA, into the *Hpa*I site of pLXSN. The erbB-4 expression vector pLXSN-erbB-4 was constructed by cloning the 4.6-kb *Sna*BI-to-*Sma*I fragment of pCH4M2 (38), which contains the complete human erbB-4 cDNA, into the *Hpa*I site of pLXSN.

Generation of recombinant Ba/F3 derivatives. Ten micrograms of a single expression vector directing the expression of an erbB family receptor or 5 μg of each of a pair of expression vectors was linearized by digestion with restriction endonucleases and ligated to form concatemers. These were electroporated into 2×10^7 Ba/F3 cells in 0.5 ml of Tris-buffered saline, using a 0.4-cm gap cuvette and a Bio-Rad Gene Pulser set at 200 V and 960 μF. Cells were immediately diluted into 50 ml of culture medium, incubated for 48 h at 37°C, and then seeded in 96-well dishes at 5×10^4 cells per well in medium supplemented with 400 μg of G418 per ml. Drug-resistant lines were expanded and screened for expression of the appropriate erbB family receptor(s). Positive lines were subcloned by limiting dilution and rescreened for receptor expression to ensure homogeneity. The cell lines characterized here are named as follows: LXSN/1 (vector control); EGFR/3; neu/5 and neu/12C; erbB-3/3; erbB-4/7; EGFR + neu/5D; EGFR + erbB-3/4A; EGFR + erbB-4/2A; neu + erbB-3/7A; neu + erbB-4/15A; and erbB-3 + erbB-4/2B.

The ranked order of receptor expression in the double recombinant cell lines is as follows. For EGFR expression, EGFR + erbB-4/2A is higher than EGFR + neu/5D, which is higher than EGFR + erbB-3/4A. For neu expression, neu + erbB-4/15A is marginally higher than EGFR + neu/5D, which is markedly higher than neu + erbB-3/7A. The levels of ErbB-3 expression are similar in the EGFR + erbB-3/4A, neu + erbB-3/7A, and erbB-3 + erbB-4/2B cell lines, while the levels of erbB-4 expression are similar in the EGFR + erbB-4/2A, neu + erbB-4/15A, and erbB-3 + erbB-4/2B cell lines.

Stimulation and analysis of erbB family tyrosine phosphorylation. A total of 2×10^8 recombinant Ba/F3 cells were washed in phosphate-buffered saline (PBS) and resuspended in 50 ml of RPMI supplemented with IL-3. The cells were incubated for 6 h at 37°C, washed in PBS, and resuspended in 1 to 2 ml of PBS. Remaining steps were performed cold or on ice. The cells were transferred in two or three 0.5- to 1.0-ml portions to microcentrifuge tubes. A chemically synthesized NRG β 65-mer peptide (1) corresponding to amino acids 177 to 241 of the NRG β1 isoform (amino acid residues are numbered according to reference 18) or the anti-neu agonistic monoclonal antibody (MAb) TAb 250 (24, 46) was added at a final concentration of 94 ng/ml (NRG) or 10 μg/ml (TAb 250). Control samples remained untreated or were treated with NRG dilution buffer. Following a 10-min incubation, cells were pelleted and incubated for 10 min in 1 ml of EBC lysis buffer (37), which is a Tris-buffered 120 mM sodium chloride solution containing 0.5% Nonidet P-40. Debris was pelleted by centrifugation, and the supernatants were transferred to a fresh tube and diluted 1:3 in EBC to facilitate sample handling. The protein content in each sample was assayed by using Coomassie blue assay reagent (Pierce), and a volume of lysate containing 2 mg of protein was used for each immunoprecipitation.

EGFR was immunoprecipitated with 900 ng of anti-EGFR MAb 528 (14) and 7.2 μg of rabbit anti-mouse antibody 31188 (Pierce); neu was immunoprecipitated with 2 μg of anti-neu MAb TAb 250 (24) and 12 μg of rabbit anti-mouse antibody or with 1 μg of anti-neu MAb FSP-16 (17) and 5 μg of rabbit anti-mouse antibody; erbB-3 was immunoprecipitated with 200 ng of anti-erbB-3 rabbit polyclonal antiserum SC-285 (Santa Cruz Biotechnology); erbB-4 was immunoprecipitated with 1 μg of anti-erbB-4 rabbit polyclonal antiserum SC-283 (Santa Cruz Biotechnology). Specificity of antireceptor antibodies was verified by testing each precipitating antibody for cross-reactivity with cell lines expressing heterologous receptors. All immunoprecipitation mixtures were incubated at 4°C for 2 h, after which the immune complexes were collected by incubation at 4°C with 50 μl of a 10% (vol/vol) suspension of fixed and washed *Staphylococcus aureus* (IGSL-10; The Enzyme Center). Immune complexes were washed three times with NET-N (37) and were eluted from *S. aureus* by boiling in 150 μl of protein sample buffer (37). Samples were divided equally, electrophoresed on separate 7.5% acrylamide–0.17% bisacrylamide–0.1% SDS gels (44), and transferred to nitrocellulose (11) for immunoblotting with either the antiphosphotyrosine MAb 4G10 (Upstate Biotechnology, Inc.) or antibodies specific for receptors. Antibody binding was detected with horseradish peroxidase-coupled sheep anti-mouse antibody NA931 (Amersham) or horseradish peroxidase-coupled donkey anti-rabbit antibody NA934 (Amersham) and enhanced chemiluminescence reagent RPN2106 (Amersham). Immunoblotting antibodies were sheep anti-EGFR polyclonal antibody 06-129 (Upstate Biotechnology Inc.), rabbit anti-sheep antibody 31240 (Pierce), rabbit anti-neu antibody Ab1 (PC04; Oncogene Science), mouse anti-erbB-3 MAb 2F12 (21), and rabbit anti-erbB-4 polyclonal antibody SC-283 (Santa Cruz Biotechnology).

RESULTS

The mouse Ba/F3 pro-B-lymphocyte cell line was chosen for expression of erbB family members because the survival and proliferation of the cells are tightly regulated by exogenous growth factor (IL-3) and because erbB family receptors have not been found to be expressed in mammalian hematopoietic cells. Immunoprecipitation and immunoblotting experiments did not reveal endogenous expression of any erbB family receptors in these cells (42). Nonetheless, we further assessed endogenous receptor expression by PCR amplification of reverse-transcribed transcripts (RT-PCR assay), the most sensitive assay available. RT-PCR analysis of erbB family receptor transcription by using probes homologous to murine erbB family receptor genes in a control Ba/F3 cell line or in Ba/F3 cell lines expressing exogenous human EGFR, neu, erbB-3, or erbB-4 demonstrated that these lines lacked endogenous murine EGFR, neu, or erbB-4 transcription (data not shown). Surprisingly, however, all of the Ba/F3 cell lines tested exhibited detectable levels of endogenous erbB-3 transcription (data not shown). This novel finding implies that erbB family receptors and their ligands may play important roles in the differentiation, expansion, or growth transformation of cells of a B-lymphocyte lineage.

cDNAs directing the expression of erbB family receptors were introduced into Ba/F3 cells to generate clonal lines that

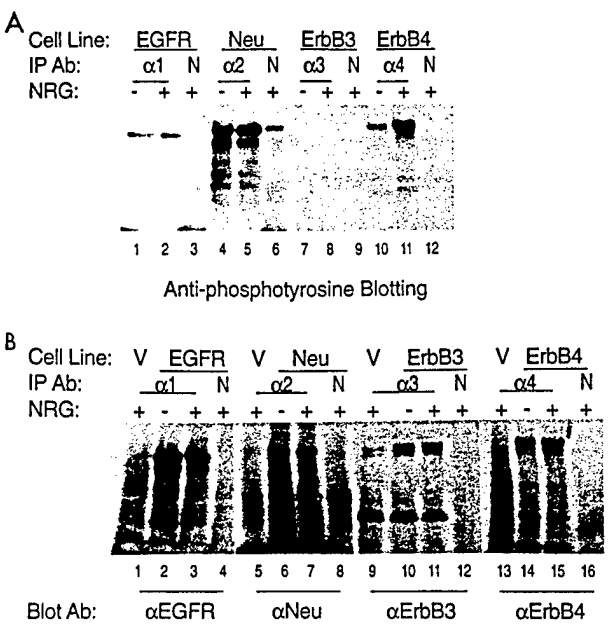

FIG. 1. Regulation of receptor tyrosine phosphorylation by NRG in single recombinant Ba/F3 derivatives. Untreated or NRG-stimulated cell lines were immunoprecipitated with antireceptor antibodies, and portions of immunoprecipitates were analyzed by immunoblotting with antiphosphotyrosine (A) or antireceptor (B) antibodies. V refers to LXSN (vector only) cells. The neu-expressing line used is neu/5. Immunoprecipitating antibodies (IP Ab): α1, anti-EGFR; α2, anti-neu; α3, anti-erbB-3; α4, anti-erbB-4; N, normal mouse or rabbit serum. +, immunoprecipitations of lysates from NRG-treated cells; −, immunoprecipitations of lysates from untreated cells.

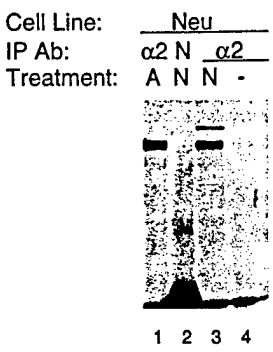

FIG. 2. Regulation of neu/12C cells by NRG. neu/12C cells were incubated with NRG dilution buffer (−), the agonistic anti-neu antibody TAb 250 (46) (A), or NRG (N). The immunoprecipitating antibody (IP Ab) used for immunoprecipitating lysates was anti-neu antibody (α2) or normal mouse serum (N); after immunoprecipitation, lysates were analyzed by immunoblotting with antiphosphotyrosine. The band above neu (lane 3) was not observed in other trials.

express the four different human receptors, singly and in combination. The resulting panel of cell lines was stimulated with a chemically synthesized NRG 65-mer peptide (amino acids 177 to 241 of NRG β1), which encompasses the EGF homology domain and is sufficient for induction of receptor tyrosine phosphorylation (1, 18). Regulation of tyrosine phosphorylation of each receptor by NRG was evaluated by immunoprecipitating the receptors and immunoblotting with antiphosphotyrosine (Fig. 1A and 3A) and antireceptor (Fig. 1B and 3B) antibodies.

Among cell lines expressing a single exogenous receptor (Fig. 1A), NRG failed to stimulate tyrosine phosphorylation of the EGFR (lanes 1 and 2) or erbB-3 (lanes 7 and 8). In contrast, NRG strongly activated tyrosine phosphorylation of erbB-4 (lanes 10 and 11). Since high basal tyrosine phosphorylation of neu in the neu/5 cell line may have obscured the effect of NRG (lanes 4 and 5), we isolated an independent Ba/F3 derivative, designated neu/12C, that expresses considerably less neu than the neu/5 cell line. In this cell line, NRG clearly activated neu tyrosine phosphorylation (Fig. 2, lanes 3 and 4).

In most of the double recombinant cell lines, NRG unambiguously stimulated tyrosine phosphorylation of both erbB family receptors (Fig. 3A; summarized in Table 1). Since the four erbB family receptors have distinct electrophoretic mobilities in most combinations, coprecipitation of heterologous dimerization partners would have been detected. However, coprecipitation was not observed under these conditions. Significantly, the results for the double recombinant cell lines are not simply additive with the responses of single cell lines. For example, NRG does not stimulate tyrosine phosphorylation of the uniquely expressed EGFR, but exogenous coexpression of erbB-3 or erbB-4 with EGFR enabled NRG to regulate EGFR tyrosine phosphorylation. Similarly, while NRG did not stimulate tyrosine phosphorylation of erbB-3 alone, coexpression of EGFR, neu, or erbB-4 permitted activation of erbB-3. Thus, NRG can regulate the tyrosine phosphorylation of each erbB family receptor provided that the appropriate coreceptor is expressed.

While NRG can stimulate the tyrosine phosphorylation of all four erbB family receptors, 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 NRG stimulation enabled survival or growth of the various Ba/F3 derivatives independent of 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 (25). Similarly, EGF stimulation of Ba/F3 cells expressing exogenous EGFR results in EGFR tyrosine phosphorylation and increased cellular DNA synthesis (4, 47), while stimulation of Ba/F3 derivatives expressing exogenous platelet-derived growth factor receptor with platelet-derived growth factor results in receptor tyrosine phosphorylation and IL-3-independent proliferation (43).

In the absence of NRG, all of the Ba/F3 derivatives, even those lines that display substantial basal receptor tyrosine phosphorylation, remained dependent on IL-3 for survival (Table 2). Likewise, NRG stimulation does not confer IL-3-independent survival or growth on control Ba/F3 cells. In cell lines expressing a single exogenous receptor, expression of neu, but not EGFR, erbB-3, or erbB-4, permitted NRG-dependent survival of Ba/F3 cells (Table 2). Indeed, all cell lines engineered to express neu (neu/5, neu/12C [42], EGFR + neu, neu + erbB-3, and neu + erbB-4) survived in the presence of NRG. This survival appears to be dependent on the amount of neu expression, as the neu/5 cell line, which expresses more neu than the other cell lines, also exhibited the strongest IL-3-independent response to NRG, while the neu/12C and neu + erbB-3 cell lines, which express less neu than the other cell lines, exhibited the weakest response to NRG treatment. NRG failed to promote the IL-3-independent survival or proliferation of erbB-4, EGFR + erbB-3, and erbB-3 + erbB-4 lines, even though NRG regulated receptor phosphorylation in these

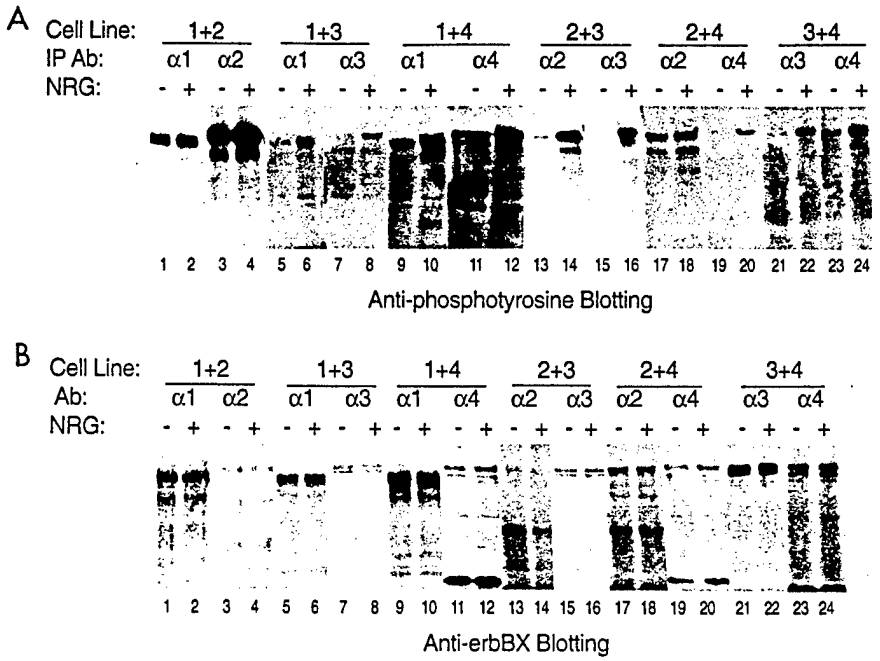

FIG. 3. Regulation of receptor tyrosine phosphorylation in double recombinant Ba/F3 derivatives. Untreated or NRG-treated cells were immunoprecipitated with antireceptor antibodies and analyzed by immunoblotting with antiphosphotyrosine (A) or antireceptor (B) antibodies. Cell lines: 1+2, EGFR + neu; 1+3, EGFR + erbB-3; 1+4, EGFR + erbB-4; 2+3, neu + erbB-3; 2+4, neu + erbB-4; 3+4, erbB-3 + erbB-4. The immunoprecipitating (IP) and/or immunoblotting antibodies (Ab): α1, anti-EGFR antibody; α2, anti-neu antibody; α3, anti-erbB-3 antibody; α4, anti-erbB-4 antibody. +, immunoprecipitations of lysates from NRG-treated cells; −, immunoprecipitations of lysates from untreated cells.

lines (Table 1). Furthermore, while NRG had no effect on the survival of cells expressing either the EGFR or erbB-4 alone, stimulation of EGFR + erbB-4 cells enabled this line to reach saturation densities comparable to those induced by IL-3 (Table 2). For this cell line, NRG acts not as an IL-3-independent survival factor but as a proliferative agent. Hence, not only is NRG-regulated receptor phosphorylation not sufficient for coupling to a cellular response, but the quality of the response is governed by the exact combination of regulated receptors present.

TABLE 1. Summary of NRG-induced erbB family receptor tyrosine phosphorylation and IL-3-independent growth or survival

Cell line	Receptor	NRG-stimulated tyrosine phosphorylation[a]	IL-3-independent growth in the presence of NRG[b]
LXSN		−	N
EGFR		−	S
neu/12C		+	S
erbB-3		−	N
erbB-4		+	N
EGFR + neu	EGFR	+/−	S
	neu	+/−	
EGFR + erbB-3	EGFR	+	N
	erbB-3	+	
EGFR + erbB-4	EGFR	+	P
	erbB-4	+	
neu + erbB-3	neu	+	S
	erbB-3	+	
neu + erbB-4	neu	+/−	S
	erbB-4	+	
erbB-3 + erbB-4	erbB-3	+	N
	erbB-4	+	

[a] Results are abstracted from Fig. 1 to 3 and similar, unpublished data. +, increased receptor tyrosine phosphorylation over basal levels; −, no increase in receptor tyrosine phosphorylation; +/−, borderline results due to high basal levels of receptor tyrosine phosphorylation.

[b] Adapted from Table 2 and similar, unpublished data. N, NRG does not enhance cell survival; S, NRG sustains viable cells; P, NRG induces IL-3-independent proliferation.

DISCUSSION

We have analyzed the NRG-dependent responses conferred upon the Ba/F3 mouse pro-B-lymphocyte cell line by expres-

TABLE 2. Ba/F3 density in response to IL-3 starvation and NRG stimulation[a]

Cell line	Viable cell saturation density (10^3 cells/ml)		
	IL-3 free	IL-3	NRG
LXSN	2	1,765	4
EGFR	1	1,301	3
neu/5	1	2,030	310
erbB-3	<1	1,800	<1
erbB-4	<1	1,583	8
EGFR + neu	2	1,104	162
EGFR + erbB-3	1	1,393	<1
EGFR + erbB-4	3	1,851	1,093
neu + erbB-3	<1	1,258	50
neu + erbB-4	1	1,664	291
erbB-3 + erbB-4	<1	1,475	3

[a] For each trial and treatment, Ba/F3 cells made quiescent by growth to saturation density were plated at a density of 100×10^3/ml in two independently seeded flasks containing medium lacking IL-3 (IL-3 free), medium supplemented with IL-3 (IL-3), or medium lacking IL-3 but supplemented with NRG β 65-mer at a final concentration of 9.4 ng/ml (NRG). Over the next 4 days, cells were stained with trypan blue and counted in a hemacytometer to determine the density of viable cells. For all treatments and cell lines, cells reached viable cell saturation densities with approximately the same kinetics. Data shown are values averaged from two or three independent trials.

sion of the four erbB family receptors, singly and in pairwise combinations. In the presence of appropriate coreceptors, 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 and can be cross-linked to neu, and binding is increased by neu overexpression (36), 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 (36), CHO cells (7, 39), T-lymphoid cells (39), or COS-7 cells (48), and NRG does not bind to solubilized neu extracellular domains (51). Moreover, NRG binds erbB-3 (2, 23, 48, 51) or erbB-4 (7, 38, 39, 51), and coexpression 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 (2, 23, 39, 48). This finding has led to the general working hypothesis that activation of neu by NRG requires the presence of erbB-3 or erbB-4.

The data presented in this report 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 (18), whereas erbB-3 binds but is impaired for kinase activity (16). 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 (Table 1). Either erbB-3 or erbB-4, both of which bind NRG, enables 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 (2), 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 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 different 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 growth and proliferation demonstrate 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 cannot 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 coreceptor 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 finding demonstrates that neu has signaling properties distinct from those of the EGFR, erbB-3, or erbB-4, consistent with earlier work showing that different erbB family receptors can activate different signaling pathways and responses (3, 10, 13, 21, 41, 49). Neither NRG nor EGF induces IL-3-independent proliferation of cells that individually express erbB-4 or EGFR (Table 2 and reference 42), 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 activated by the EGFR and one activated by erbB-4. An interesting alternative would be that EGFR and erbB-4 phosphorylation sites are different 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. Preliminary evidence supports this prediction. Three different members of the EGF family induce different patterns of erbB family receptor tyrosine phosphorylation and IL-3-independent growth in the Ba/F3 derivatives described here (42). Given the multitude of roles that erbB family receptors and their ligands apparently play in diverse biological processes such as neurogenesis, neuromuscular signaling, tumorigenesis, wound healing, and the regulation of mesenchymal-epithelial cell interactions, it is likely that all of the mechanisms described here play a significant part in specifying responses to ligand stimulation in vivo.

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Betacellulin activates the epidermal growth factor receptor and erbB-4, and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neuregulin-β

David J Riese II[1], Yamilée Bermingham[1], Tom M van Raaij[1], Sharon Buckley[2], Gregory D Plowman[2], and David F Stern[1]

[1]*Department of Pathology, Yale University School of Medicine, New Haven, CT 06520-8023, USA;* [2]*Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Washington, 98121, USA, and Sugen, Inc., 515 Galveston Drive, Redwood City, CA 94063-4720, USA*

Betacellulin is a member of the epidermal growth factor (EGF) family. These soluble proteins are ligands for one or more of 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). While evidence suggests that betacellulin is a ligand for the EGFR, the ability of betacellulin to regulate other erbB family receptors has not been analysed. Previously we engineered derivatives of the mouse Ba/F3 hematopoietic cell line to ectopically express erbB family receptors, singly and in pairwise combinations. We have stimulated this panel of cell lines with betacellulin and two other EGF family members, EGF itself and neuregulin-β (NRG-β). In the cell lines expressing a single erbB family receptor, betacellulin not only stimulated EGFR tyrosine phosphorylation, but it activated erbB-4 as well. Furthermore, in the double recombinant Ba/F3 derivatives, betacellulin stimulated a complex pattern of receptor phosphorylation distinct from the patterns activated by NRG-β and EGF. Moreover, betacellulin stimulated a complex pattern of interleukin-3 independence in the Ba/F3 derivatives distinct from those activated by NRG-β and EGF. These data identify a novel receptor for betacellulin and establish that different EGF family ligands activate distinct patterns of receptor phosphorylation and coupling to cellular signaling pathways.

Keywords: betacellulin; EGF; neuregulin; EGF receptor; ErbB receptors; signaling

Introduction

The epidermal growth factor (EGF) family consists of at least 15 members, including epidermal growth factor, transforming growth factor alpha (TGFα), amphiregulin (AR), betacellulin, heparin-binding-EGF-like growth factor (HB-EGF), cripto, epiregulin and the several differentially-spliced variants of neuregulin (NRGs), also known as heregulins, neu differentiation factors, gp30, glial growth factors and acetylcholine receptor inducing activity (Reviewed in Groenen, *et al.*, 1994). These factors activate a family of four receptor tyrosine kinases encoded by the erbB gene family (erbB-1/epidermal growth factor receptor (EGFR), neu/erbB2/HER2, erbB-3/HER3, and erbB-4/HER4). The patterns of receptor activation stimulated by EGF family ligands is very complex; some of these peptides bind to and activate signaling by more than one erbB family receptor. Furthermore, in cell lines expressing multiple erbB receptor family members, these ligands can activate erbB family receptors that do not bind these ligands when the receptors are expressed individually, a process called transmodulation (Reviewed in Hynes and Stern, 1994; Earp *et al.*, 1995). For example, EGF activates neu when coexpressed with the EGFR, but EGF does not bind or activate neu expressed on its own (Akiyama, *et al.*, 1988; King *et al.*, 1988; Stern and Kamps, 1988; Connelly and Stern, 1990). It has been proposed that transmodulation 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). Not only does EGF induce erbB receptor transmodulation, but every EGF family member tested thus far has this activity (Stern and Kamps, 1988; Johnson *et al.*, 1993; Plowman *et al.*, 1993; Carraway *et al.*, 1994; Kita *et al.*, 1994; Sliwkowski *et al.*, 1994; Karunagaran *et al.*, 1995; Riese *et al.*, 1995).

Betacellulin was initially identified as a factor in the conditioned medium of a mouse pancreatic β cell carcinoma (insulinoma) cell line that is mitogenic for Balb/C 3T3 cells. The 80 amino acid mature betacellulin protein is derived from a 177 amino acid membrane-bound precursor and contains the six conserved cysteine residues that are arranged in a characteristic pattern common to all of the EGF family members. Furthermore, betacellulin has significant overall homology to mature EGF, TGF-α, AR, HB-EGF, and NRGs (Sasada *et al.*, 1993; Shing *et al.*, 1993; Reviewed in Groenen *et al.*, 1994). Binding of human recombinant betacellulin to the A431 human adenocarcinoma cell line, which overexpresses the EGFR, can be competed with an excess of EGF, suggesting that betacellulin is a ligand for the EGFR (Watanabe *et al.*, 1994). However, previous studies have not assessed betacellulin binding to other erbB family receptors or betacellulin stimulation of erbB family receptor tyrosine phosphorylation or coupling to cellular signaling pathways.

Little is known about the *in vivo* functions of betacellulin. While its expression in the BTC-3 mouse insulinoma cell line (Shing *et al.*, 1993) and the MCF-7 human breast adenocarcinoma cell line (Sasada *et al.*, 1993) implies that betacellulin regulates the prolifera-

Correspondence: DF Stern
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tion of pancreatic and breast cells and may play a causative role in breast and pancreatic cancer, the receptor(s) for betacellulin must be identified before definitive studies of the physiologic effects of betacellulin can be undertaken. In this study we have used derivatives of the Ba/F3 mouse pro-B lymphocyte cell line that have been engineered to ectopically express, singly and in pairwise combinations, all four erbB family receptors (Riese *et al.*, 1995). We have assessed erbB family receptor tyrosine phosphorylation and the biological responses of these cell lines to stimulation with betacellulin and two other EGF family ligands, neuregulin-β (NRG-β) and EGF itself. Not only do these experiments identify a novel receptor for betacellulin, but we also demonstrate that betacellulin stimulates complex patterns of erbB family receptor tyrosine phosphorylation and coupling to cellular signaling pathways that are unique from the patterns stimulated by EGF and NRG-β.

Results

erbB family receptor tyrosine phosphorylation in single recombinant cell lines

The Ba/F3 cell line is a mouse pro-B lymphocyte cell line (Palacios and Steinmetz, 1985) that does not express endogenous EGFR, neu and erbB-4, but does express low levels of erbB-3 (Riese *et al.*, 1995). We have previously described the generation of Ba/F3 derivatives that ectopically express the four human erbB family receptors, singly and in all pairwise combinations (Riese *et al.*, 1995). In this report, we have stimulated this panel of cell lines with recombinant human betacellulin, NRG-β and EGF and assessed erbB family receptor tyrosine phosphorylation. The recombinant human betacellulin used in these experiments consisted of 34 amino acids of the human amphiregulin precursor (Val107–Arg140), linked to the 50 amino acid EGF-structural motif of human betacellulin (Arg31–Tyr80) and a 9 amino acid hemagglutinin epitope. The amphiregulin sequences in this molecule are not within the EGF-structural motif and therefore are not predicted to contribute to receptor binding. Furthermore, this recombinant betacellulin produced as a tagged fusion construct in bacteria had an activity indistinguishable from that produced transiently in COS cells using an expression construct containing the full length human betacellulin sequence (Plowman *et al.*, in preparation).

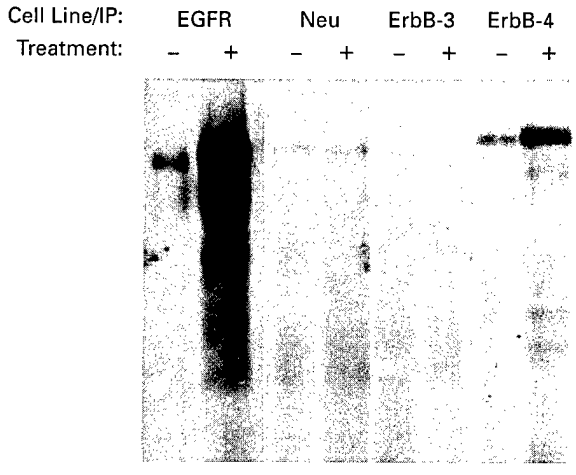

Figure 1 Regulation of receptor tyrosine phosphorylation by betacellulin in single recombinant Ba/F3 derivatives. Lysates from untreated or betacellulin-treated Ba/F3 derivatives were immunoprecipitated with anti-receptor antibodies and analysed by immunoblotting with anti-phosphotyrosine antibody. The cell lines and immunoprecipitating antibodies are as marked. Lysates from betacellulin-treated cells are denoted '+' while lysates from untreated cells are denoted '–'

In cell lines that ectopically express a single erbB family receptor, both betacellulin and EGF stimulated EGFR tyrosine phosphorylation (Figures 1 and 2a). Surprisingly, betacellulin (as well as NRG-β) stimulated erbB-4 tyrosine phosphorylation (Figures 1 and 2a). Not only do these data demonstrate that betacellulin regulates EGFR signaling, but they also indicate that erbB-4 is a receptor for betacellulin. Betacellulin did not stimulate increased neu or erbB-3 tyrosine phosphorylation (Figures 1 & 2a), suggesting that neither neu nor erbB-3 is a receptor for betacellulin (NRG-β stimulation of neu tyrosine phosphorylation is probably due to endogenous erbB-3 expression (Riese, *et al.*, 1995). However, because erbB-3 has only minimal intrinsic tyrosine kinase activity (Guy *et al.*, 1994), these data do not rule out the possibility that erbB-3 can bind betacellulin.

Since some AR sequences were present in the recombinant betacellulin used in these experiments, there was a remote possibility that these contributed to the unanticipated activation of erbB-4. Hence, we compared the activities of our betacellulin preparation with those of a recombinant mature-form preparation comprised of betacellulin exclusively (R and D Systems). In the cell line expressing erbB-4, both betacellulin preparations stimulated similar maximal levels of erbB-4 tyrosine phosphorylation at 3 nM betacellulin (data not shown). This maximal level was similar to that stimulated by 6 nM NRG-β (data not shown). Therefore, stimulation of EGFR and erbB-4 phosphorylation by betacellulin was not conferred by AR sequences.

erbB family receptor tyrosine phosphorylation in double recombinant cell lines

In activating both the EGFR and erbB-4, betacellulin displays activities distinct from EGF, which activates the EGFR alone, and NRG-β, which binds to erbB-3 and erbB-4. We next compared the effects of betacellulin, EGF, and NRG-β on receptor transmodulation by assessing receptor tyrosine phosphorylation in cell lines expressing combinations of erbB family receptors. Betacellulin, as well as EGF, stimulated the tyrosine phosphorylation of both receptors in the EGFR + neu (1+2), EGFR + erbB-3 (1+3), and EGFR + erbB-4 (1+4) cell lines (Figure 2b and c). Therefore, both betacellulin and EGF can transmodulate the other three receptors when co-expressed with EGFR. However, the three ligands did not stimulate equal levels of receptor phosphorylation. In 1+3 cells, all three ligands stimulated approximately equal levels of erbB-3 phosphorylation, while betacellulin and EGF stimulated higher levels of EGFR phosphorylation than NRG-β did (Figure 2b). Similarly, in 1+4 cells,

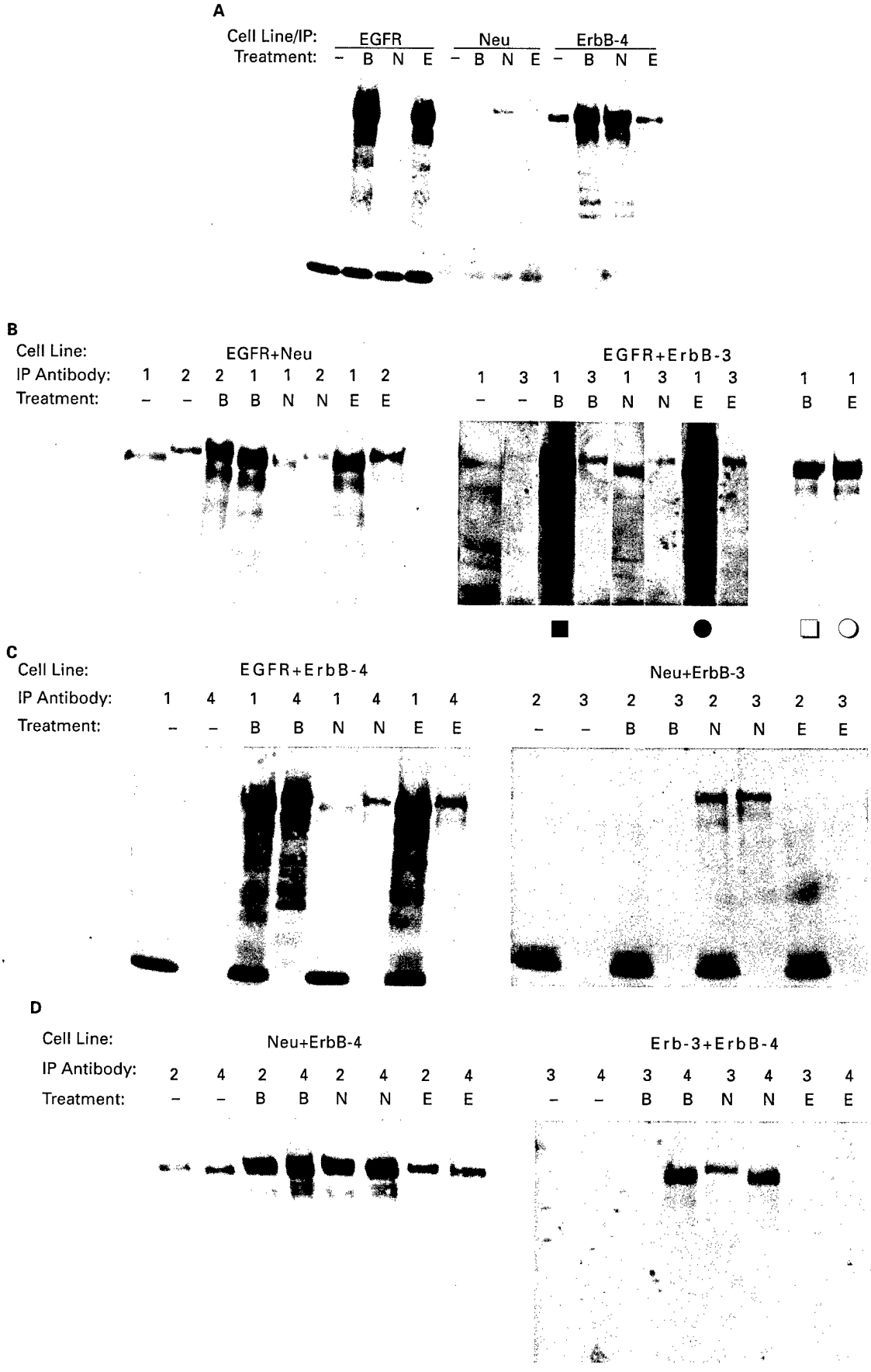

Figure 2 (**A**–**D**) Comparison of receptor tyrosine phosphorylation in Ba/F3 derivatives stimulated with betacellulin, EGF, or NRG-β. Lysates from untreated or ligand-treated Ba/F3 derivatives were immunoprecipitated with anti-receptor antibodies and analysed by immunoblotting with anti-phosphotyrosine antibody. The cell lines are as marked. Immunoprecipitating antibodies were: 1, anti-EGFR; 2, anti-Neu; 3, anti-erbB-3; 4, anti-erbB-4. Lysates from betacellulin-treated cells are denoted 'B', lysates from NRG-β-treated cells are denoted 'N', lysates from EGF-treated cells are denoted 'E' and lysates from untreated cells are denoted '–'. Lighter exposures of the lanes denoted by ■ and ● are denoted by □ and ○

betacellulin stimulated approximately equal levels of EGFR and erbB-4 phosphorylation, while EGF stimulated higher levels of EGFR phosphorylation than erbB-4 phosphorylation and NRG-β stimulated higher levels of erbB-4 phosphorylation than EGFR phosphorylation (Figure 2c). One explanation for these differences is that EGFR, erbB-3, and erbB-4 may have a lower affinity for heterotypic interactions and transmodulation than for the homotypic interactions induced by direct stimulation.

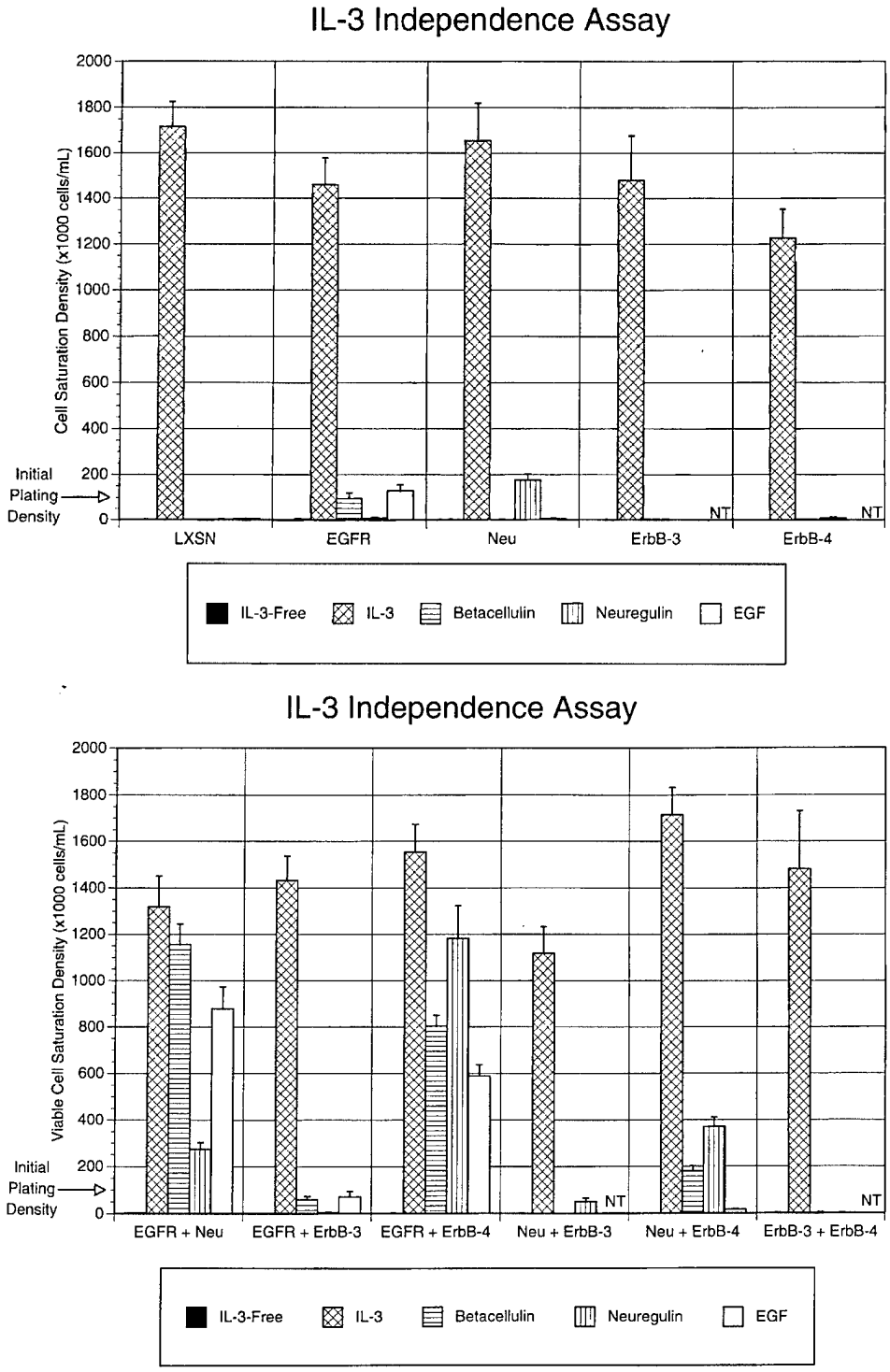

Figure 3 IL-3-independent saturation density of Ba/F3 cells treated with betacellulin, EGF, or NRG-β. Ba/F3 derivatives were plated at a density of 100×10^3 cells/ml in medium lacking IL-3 (IL-3-Free), medium supplemented with IL-3 (IL-3), or in medium lacking IL-3 but supplemented with 10 ng/ml human recombinant EGF (EGF), 9.4 ng/ml NRG-β 65- mer (Neuregulin), or 7 ng/ml human recombinant betacellulin (Betacellulin). Viable cell saturation densities were calculated 2 to 4 days after seeding. Each combination of cell lines and factors was tested 4 to 20 times and the arithmetic means of the viable cell saturation densities are indicated by the filled bars, while the standard error of the means are indicated by the error bars. Because cultures treated with IL3-free medium exhibited densities of less than 4×10^3 cells/ml, these values, represented by the left-most bar for each cell line, are not apparent on the graphs. Some of the data describing the responses to stimulation with NRG-β or control media has been previously published (Riese *et al.*, 1995). 'NT' indicates not tested

As expected from the response of the erbB-4 cell line, betacellulin and NRG-β stimulated erbB-4 tyrosine phosphorylation in the three double recombinant cell lines that express erbB-4. However, while both betacellulin and NRG-β stimulated EGFR and neu tyrosine phosphorylation in the EGFR + erbB-4 (1+4) and neu + erbB-4 (2+4) cell lines, respectively, betacellulin did not stimulate erbB-3 tyrosine phosphorylation in the erbB-3 + erbB-4 (3+4) cell line, even through NRG-β did (Figures 2c and d). Finally, betacellulin did not stimulate phosphorylation of either receptor in neu + erbB-3 cells, while NRG-β did (Figure 2c), suggesting that neither neu nor erbB-3 is a receptor for betacellulin.

IL-3-independent growth of Bal/F3 derivatives

We next tested whether betacellulin, NRG-β, and EGF stimulate different patterns of receptor coupling to cellular signaling pathways. Since the recombinant Ba/F3 cell lines are strictly dependent upon interleukin-3 (IL-3) for survival and proliferation (Riese *et al.*, 1995), we tested whether betacellulin, NRG-β, or EGF induced the IL-3-independent survival or proliferation of the various Ba/F3 derivatives. Activation of either EGFR or neu in the single recombinant cell lines was associated with IL-3 independent survival but not proliferation (Figure 3a), while activation of erbB-3 or erbB-4 in the single recombinants had no biological effect (Figure 3a). 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 (Figure 3b). 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 the 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. The exception is the response of EGFR + erbB-3 (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, despite stimulating both EGFR and erbB-3 tyrosine phosphorylation in this cell line (Figure 2b).

Furthermore, in some of the double recombinant cell lines ligand stimulation of coupling of multiple receptors to signaling pathways acted in a nonadditive manner to stimulate an IL-3 independent response (Figure 3b). In 1+2 cells betacellulin and EGF stimulated IL-3 independent proliferation, while in 1+4 cells betacellulin and NRG-β stimulated IL-3 independent proliferation and EGF stimulated a response intermediate to survival and proliferation. Therefore, while activation of either EGFR alone or neu alone stimulated IL-3 independent survival, in some cases activation of EGFR along with either neu or erbB-4 conferred IL-3 independent proliferation.

Discussion

Previous reports demonstrated that betacellulin binds to the A431 human adenocarcinoma cell line, which overexpresses 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 (Table 1). This is consistent with the

Table 1 Summary of stimulation of receptor tyrosine phosphorylation and IL-3 independence

Cell line	*Receptor*	*Betacellulin tyr phos.*[a]	*Betacellulin IL-3 indpt.*[b]	*NRG-β tyr phos.*[a]	*NRG-β IL-3 indpt.*[b]	*EGF tyr phos.*[a]	*EGF IL-3 indpt.*[b]
EGFR		+	S	–	N	+	S
Neu		–	N	+[c]	S[c]	–	N
erbB-3		–	N	–	N	–	NT[d]
erbB-4		+	N	+	N	–	NT[d]
EGFR + Neu	EGFR	+	P	*[c]	S[c]	+	P
	Neu	+		*[c]		+	
EGFR + erbB-3	EGFR	+	S	+	N	+	S
	erbB-3	+		+		+	
EGFR + erbB-4	EGFR	+	P	+	P	+	S/P
	erbB-4	+		+		+	
Neu + erbB-3	Neu	–	N	+	S	–	NT[d]
	erbB-3	–		+		–	
Neu + erbB-4	Neu	+	S	+	S	–	N
	erbB-4	+		+		–	
erbB-3 + erbB-4	erbB-3	–	N	+	N	–	N
	erbB-4	+		+		–	

[a] Results are abstracted from Figures 1 and 2, Riese *et al.*, 1995, and similar unpublished data. '+' indicates increased receptor tyrosine phosphorylation over basal levels, '–' indicates no increase in receptor tyrosine phosphorylation, and '*' indicates ambiguity due to high basal levels of receptor phosphorylation. [b] Results are abstracted from Figure 3. 'N' indicates no IL-3 independent response, 'S' indicates stimulation of IL-3 independent survival, 'P' indicates stimulation of IL-3 independent proliferation, and 'S/P' indicates stimulation of an intermediate response. 'NT' indicates not tested. [c] The response to NRG is apparently due to interactions with the endogenous erbB-3 in Ba/F3 cells (Riese *et al.*, 1995). [d] Given the absence of receptor tyrosine phosphorylation, no IL-3 independent response is expected

observation that radiolabeled betacellulin binds specifically to EGFR and erbB-4, but not to neu (Plowman *et al.*, in preparation). 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 (Table 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 (Table 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 (Table 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 trosine phosphorylation of both receptors, while betacellulin stimulates the tyrosine phosphorylation of erbB-4 but not of erbB-3 (Table 1). Nonetheless, because NRG-β binds erbB-3, it is possible that this absence of erbB-3 tyrosine phosphorylation may not be 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 IL3 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).

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 (Table 1). 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 (Figure 2b). 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 multiple receptor species to cellular signaling pathways acts in a non-additive manner in specifying biological responses (Riese *et al.*, 1995). 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).

Biological responses to EGF family ligands are regulated by several hierarchical mechanisms (Riese *et al.*, 1995). 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, 1993). 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 FGFRsand Trks can bind multiple ligands (Reviewed in Johnson and Williams, 1993; 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,b). 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 properties 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, 1993; 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 increases in the expression and/or signaling of erbB family receptors plays a significant role in tumors of mammary or neuroectodermal origin (Reviewed in Hynes and Stern, 1994). Because betacellulin, NRG-β and EGF have distinct biological activities that apparently reflect their differing abilities to activate receptor signaling, it may be possible to develop antagonists that specifically disrupt signaling by a single EGF family ligand and may inhibit the genesis or growth of malignancies without disrupting the activity of other EGF family ligands in the same tissue.

Materials and methods

Production of human recombinant Betacellulin

To facilitate refolding and purification, human recombinant betacellulin was produced as an epitope-tagged fusion with human amphiregulin. The pPL-Lambda (Pharmacia) thermoinducible bacterial expression vector was modified to remove the EcoRI, BamHI and SmaI sites upstream from the PL promoter and a human betacellulin transcription unit was inserted into the unique HpaI site within the N gene. The insertion contained the lac and Cro gene Shine-Delgarno ribosome binding sites; a unique BglII cloning site; an initiating methionine codon; the nucleotide sequence encoding 34 amino acids of the human amphiregulin precursor (Val107–Arg140), the 50 amino acid EGF-structural motif of human betacellulin (Arg31–Tyr80) and a 9 amino acid hemagglutinin epitope sequence (PYDVPDYAS); a stop codon; unique EcoRV and XbaI restriction sites; and transcription termination sequences. The resulting plasmid, pPLABTC-Tag, was transformed into competent *E. Coli* N4830-1 and grown at 30°C in 1 litre LB media with 50 ug/ml ampicillin to an Abs_{600} of 0.7. Cultures were then induced by incubation at 42°C for 18–24 h. Following induction, cells were harvested by centrifugation at 5000*g*, washed in STE buffer (50 mM Tris, pH 8.0/200 mM NaCl/2 mM EDTA). The pellet was resuspended in STE containing 2 mM 2-mercaptoethanol, and lysed by addition of 0.2 mg/ml lysozyme followed by addition of Triton X-100 and Zwittergent (CalBiochem) to 1%. To ensure lysis and solubilization of non-inclusion body protein, the preparation was sonicated for 2 min and centrifuged at 13 000*g* for 10 min. The pellet was resuspended in STE and sonicated again for an additional 1 min. The slurry was then layered on a 40% sucrose cushion and centrifuged at 13 000*g* for 10 min at 4°C. The inclusion body pellet was resuspended in 6 M guanidine-HCl (GuHCl)/50 mM CAPS, pH 11.0.

The Betacellulin inclusion body preparation was diluted to 60 mM GuHCl with 50 mM CAPS, pH 11.0/1 mM EDTA/1.25 mM reducing glutathione/0.5 mM oxidizing glutathione. The final protein concentration was 50–100 mg/ml by Biorad protein assay. Refolding was achieved by incubation at 4°C for 18–24h. The solution was then dialyzed against 50 mM sodium phosphate (NaP), pH 7.5, and successively filtered through 5 mm, 0.45 mm and 0.22 mm filters or subjected to 60 000*g* centrifugation prior to cation exchange chromatography. Alternatively, the refolded material was buffer exchanged by ultrafiltration against 3 volumes of 50 mM NaP, pH 7.5.

Cleared, refolded bacterially-produced betacellulin was loaded on a cation exchange column (Bakerbond CSx) equilibrated with 40 mM NaP pH 7.0. The flow rate was 1.25 ml/min and the chromatography was carried out at room temperature. The column was washed with 20 column volumes of 40 mM NaP, pH 7.0, or until a stable baseline was achieved. The betacellulin was eluted with a 50 ml linear gradient of 0.2 – 1 M NaCl in 40 mM NaP, pH 7.0. The peak fractions were at ~550 mM NaCl as determined by reactivity in a hemagluttinin ELISA. The peak fractions from cation exchange chromatography were pooled, diluted to 0.2 M NaCl with 40 mM NaP, pH 7.0 and applied to an FPLC TSK-heparin 5PW column (TosoHaas). The flow rate was 1 ml/min. The column was then washed with 40 mM NaP, pH 7.0 and bound protein was eluted with a 30 ml linear gradient of 0–1.0 M NaCl in 40 mM NaP, pH 7.0. The recombinant tagged betacellulin eluted at 800 mM NaCl and migrated as a single Coomassie stained band on 15% SDS-PAGE.

Betacellulin activity was measured using an EGFR tyrosine phosphorylation assay (Thorne and Plowman, 1994). Recombinant betacellulin produced as a tagged fusion construct in bacteria had an activity indistinguishable from that produced transiently in COS cells using an expression construct containing the full length human betacellulin sequence (Plowman *et al.*, in preparation).

Cell lines and cell culture

cDNAs encoding the four human erbB family receptors were subcloned into the recombinant retroviral expression vector pLXSN (Miller and Rosman, 1989), which carries a neomycin resistance gene. pLXSN and the constructs expressing the erbB family receptors were transfected into the mouse Ba/F3 pro-B-lymphocyte cell line (Palacios and Steinmetz, 1985) and selected with geneticin, generating a vector control cell line as well as clonal cell lines that express the four erbB family receptors, singly and in pairwise combinations. The generation of these cell lines has been described previously (Riese *et al.*, 1995). The cell lines used in this report are: LXSN/1 (vector control); EGFR/3; neu/12C; erbB-3/3; erbB-4/7; EGFR + neu/5D; EGFR + erbB-3/4A; EGFR + erbB-4/2A; neu + erbB-3/7A; neu + erbB-4/15A; and erbB3 + erbB-4/2B. Cells were grown in RPMI (Gibco/BRL) supplemented with 10% fetal calf serum (Sigma), 200 ug/ml G418 (Gibco/BRL), and interleukin-3 (IL-3) supplied as 10% conditioned

medium from the WEHI-3B mouse myelomonocytic leukemia cell line (Daley and Baltimore, 1988).

The ranked order of receptor expression in the double recombinant cell lines is as follows. For EGFR expression, EGFR + erbB-4/2A is higher than EGFR + neu/5D, which is higher than EGFR + erbB-3/4A. For neu expression, neu + erbB4/15A is marginally higher than EGFR + neu/5D, which is markedly higher than neu + erbB-3/7A. The levels of ErbB-3 expression are similar in the EGFR + erbB3/4A, neu + erbB-3/7A, and erbB-3 + erbB-4/2B cell lines, while the levels of erbB-4 expression are similar in the EGFR + erbB-4/2A, neu + erbB-4/15A and erbB-3 + erbB-4/2B cell lines (Riese *et al.*, 1995).

Stimulation and analysis of erbB family receptor tyrosine phosphorylation

2×10^8 recombinant Ba/F3 cells were washed in phosphate-buffered saline (PBS) and resuspended in 50 ml RPMI supplemented with IL-3. The cells were incubated for 6–10 h at 37°C, washed in ice-cold PBS and resuspended in 1–3 ml ice-cold PBS. Remaining steps were performed cold or on ice. The cells were transferred in two to five 0.5 ml portions to microcentrifuge tubes. Human recombinant EGF (Collaborative Biomedical), a chemically-synthesized NRG-β 65mer (Barbacci *et al.*, 1995; Riese *et al.*, 1995) or recombinant human betacellulin was added at a final concentration of 100 ng/ml (EGF), 94 ng/ml (NRG-β), or 150 ng/ml (betacellulin). Following a 10 min incubation, cells were lysed and the protein content of each sample was assayed as described earlier (Riese *et al.*, 1995).

Analysis of erbB family receptor tyrosine phosphorylation has been described previously (Riese *et al.*, 1995). Briefly, the erbB family receptors were immunoprecipitated with antisera specific for single receptors, after which the samples were separated by electrophoresis through a 7.5% acrylamide, 0.17% bisacrylamide, 0.1% SDS gel (Sefton *et al.*, 1979), electrotransferred onto nitrocellulose (DiGiovanna and Stern, 1995) and immunoblotted with the antiphosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc.). Antibody binding was detected with sheep anti-mouse coupled to horseradish peroxidase antibody NA931 (Amersham) and enhanced chemiluminescence reagents RPN2106 (Amersham).

Anti-receptor antibodies used for immunoprecipitation were as follows: antiEGFR mouse monoclonal antibody 528 (Gill *et al.*, 1984); anti-Neu mouse monoclonal antibodies TAb 250 (Langton, *et al.*, 1991), FSP16 (Harwerth *et al.*, 1992) and TA-1 (Ab-5, Oncogene Science); anti-erbB-3 rabbit polyclonal antiserum SC-285 (Santa Cruz Biotechnology); and anti-erbB-4 rabbit polyclonal antiserum SC-283 (Santa Cruz Biotechnology). Specificity of anti-receptor antibodies has been verified previously by testing for cross-reactivity with lysates from cell lines expressing heterologous receptors (data not shown).

Stimulation and analysis of IL-3 independence

For each trial and treatment, Ba/F3 derivatives made quiescent by growth to saturation density were plated at a density of 100×10^3 cells/ml in duplicate culture dishes containing medium lacking IL-3 (IL-3-Free), medium supplemented with IL-3 (IL-3), or in medium lacking IL-3 but supplemented with 10 ng/ml human recombinant EGF (EGF–Collaborative Biomedical), 9.4 ng/ml chemically-synthesized NRG-β 65- mer (Neuregulin–(Barbacci, *et al.*, 1995; Riese *et al.*, 1995)), or 7 ng/ml human recombinant betacellulin (Betacellulin). Cells were stained daily with trypan blue and counted in a hemacytometer to determine viable cell densities until each sample reached a viable cell saturation density, at which time data collection was terminated. While not every cell line was tested with every factor in every trial, each combination of cell lines and factors shown was tested in a minimum of 4 trials, while some combinations were tested in as many as 20 trials. The arithmetic means and the standard error of the means of the viable cell saturation densities were calculated for each combination of cell line and treatment (Zar, 1984). Cultures exhibiting viable cell saturation densities of less than 20×10^3 cells/ml were judged to be nonresponsive to stimulation. Cultures with viable cell saturation densities of $50-400 \times 10^3$ cells/ml were judged to be exhibiting survival but not proliferation. Cultures with viable cell saturation densities of greater than 800×10^3 cells/ml were judged to be exhibiting proliferation.

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The Epidermal Growth Factor Receptor Couples Transforming Growth Factor-α, Heparin-binding Epidermal Growth Factor-like Factor, and Amphiregulin to Neu, ErbB-3, and ErbB-4*

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David J. Riese II‡§, Edward D. Kim‡, Klaus Elenius¶‖, Sharon Buckley‡, Michael Klagsbrun¶‡‡, Gregory D. Plowman**, and David F. Stern‡§§**

*From the ‡Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06520-8023, ¶Department of Surgical Research, Children's Hospital and Harvard Medical School, Boston, Massachusetts 02115-5746, **Bristol-Myers-Squibb Pharmaceutical Research Institute, Seattle, Washington 98121, and Sugen, Inc., Redwood City, California 94063-4720*

The epidermal growth factor (EGF) family hormones amphiregulin (AR), transforming growth factor-α (TGF-α), and heparin-binding EGF-like growth factor (HB-EGF) are thought to play significant roles in the genesis or progression of a number of human malignancies. However, the ability of these ligands to activate all four erbB family receptors has not been evaluated. Therefore, we have assessed the stimulation of erbB family receptor tyrosine phosphorylation by these hormones in a panel of mouse Ba/F3 cell lines expressing the four erbB family receptors, singly and in pairwise combinations. We also measured the stimulation of interleukin-3-independent survival or proliferation in this panel of Ba/F3 cell lines to compare the patterns of erbB family receptor coupling to physiologic responses induced by these peptides. EGF, TGF-α, AR, and HB-EGF all stimulated qualitatively similar patterns of erbB family receptor tyrosine phosphorylation and coupling to physiologic responses. Therefore, EGF, TGF-α, AR, and HB-EGF are functionally identical in this model system and behave differently from the EGF family hormones betacellulin and neuregulins.

Deregulation of the signaling network composed of the erbB family receptors and the epidermal growth factor (EGF)[1] family of peptide hormones plays an important role in a number of human metastatic diseases (reviewed in Ref. 1). Several factors contribute to the complex regulation of this system. There are four distinct erbB family receptors, including the epidermal growth factor receptor (EGFR/erbB-1), neu (erbB-2/HER2), erbB-3 (HER3), and erbB-4 (HER4). Receptor signaling can be stimulated by at least six different EGF family hormones, including EGF, transforming growth factor-α (TGF-α), amphiregulin (AR), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), and the several differentially spliced variants of neuregulin (NRG), also known as neu differentiation factors, heregulins, glial growth factors, or acetylcholine receptor inducing activity (reviewed in Refs. 1–3). A single erbB family receptor (*e.g.* EGFR) can bind several different EGF family hormones (EGF, TGF-α, AR, HB-EGF) (reviewed in Refs. 1 and 2), and a single EGF family peptide (BTC) can activate more than one receptor (EGFR, erbB-4) (4). Furthermore, in cells expressing multiple erbB family receptors, EGF family peptides can induce the phosphorylation and signaling of erbB family receptors that, when expressed alone, are not phosphorylated, a process referred to as transmodulation (reviewed in Refs. 5 and 6). For example, EGF stimulates neu tyrosine phosphorylation when neu is coexpressed with the EGFR, but not when neu is expressed by itself (7–10). Indeed, with a single exception, every EGF family hormone tested thus far has the potential to regulate all four erbB family receptors through this mechanism (4, 7–19). The physiological relevance of transmodulation is supported in gene-targeting experiments in transgenic mice. Mice homozygous for disruptions in the NRG, neu, or erbB-4 genes all die *in utero* at day 10.5 and lack the trabecular extensions of the developing ventricular myocardium (20–22). The similarity of these cardiac defects suggests that NRG induction of both neu and erbB-4 signaling is required for cardiac morphogenesis. Since NRG regulates neu phosphorylation only through transmodulation (12–15, 17–19), erbB-4-dependent transmodulation of neu signaling by NRG plays a critical role in cardiac development.

Despite the relevance of erbB family receptor phosphorylation and transmodulation in regulating cellular proliferation and differentiation, the effects of TGF-α, AR, and HB-EGF on erbB family receptor signaling have not been completely evaluated. All three of these molecules stimulate EGFR phosphorylation (11, 23, 24), and both AR and TGF-α transmodulate neu (9, 11). However, the abilities of these molecules to regulate erbB-3 and erbB-4, alone and in combination with other receptors, have not been comprehensively evaluated. Consequently, while all three of these molecules stimulate EGFR signaling, they may also differentially regulate signaling by the other three erbB family receptors. This might explain quantitative differences observed in the activities of AR, HB-EGF,

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§§ To whom correspondence should be addressed: Dept. of Pathology, Yale University School of Medicine, P. O. Box 208023, New Haven, CT 06520-8023. Tel.: 203-785-4832; Fax: 203-785-7467; E-mail: Stern@Biomed.med.yale.edu.

[1] The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; AR, amphiregulin; NRG, neuregulin; TGF-α, transforming growth factor-α; HB, heparin binding; BTC, betacellulin; IL, interleukin; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

and EGF (25, 26). This possibility is underscored by our recent finding that BTC not only activates EGFR, but also activates erbB-4 (4). In this report we have evaluated AR, HB-EGF, and EGF activities by stimulating IL-3-dependent Ba/F3 mouse pro-B-lymphocyte cell lines that have been engineered to express, singly and in pairwise combinations, all four erbB family receptors (4, 17).

EXPERIMENTAL PROCEDURES

Growth Factors—The production of recombinant HB-EGF has been described earlier (27). Human recombinant EGF and TGF-α were supplied by Collaborative Biomedical Products. NRG was supplied as a chemically synthesized NRG-β 65-mer (17, 28). Amphiregulin purification took advantage of the highly basic nature and high heparin affinity of AR. Due to the high isoelectric point (pI 10.1) of nonglycosylated AR, we compared AR refolding efficiency over a range of pH 7–11. Maximal activity was seen following refolding in 50 mM CAPS, pH 11.0.

AR was produced in bacteria under the control of the tightly regulated, thermoinducible bacteriophage λ PL promoter as described for betacellulin (4) with several modifications. The nucleotide sequence encoding an initiating methionine, 82 amino acids of the AR precursor (Val^{107}–Thr^{188}), and a stop codon was inserted between the unique *Bgl*II and *Xba*I sites of pPLABTC-Tag (4). The resulting plasmid was grown and transformed into competent *Escherichia coli* N4830-1. AR expression was induced and bacteria were lysed and fractionated as described previously (4). Recombinant AR was refolded in 50 mM CAPS at pH 11.0, followed by cation exchange and heparin affinity chromatography as described previously (4). AR activity was monitored using an EGFR tyrosine phosphorylation assay and an AR-specific enzyme-linked immunosorbent assay (29).

The nonglycosylated bacterial AR migrated at 14 kDa, consistent with the *N*-glycanase-treated native AR. Furthermore, it was found to inhibit the binding of ^{125}I-EGF to NRHER5 membranes (which express EGFR) as well as to live cells (29). A 50% inhibition of ^{125}I-EGF binding to NRHER5 membranes was seen at approximately 0.1 nM EGF (0.1 ng/well), 100 nM native AR or recombinant AR produced in COS cells (100 ng/well), and 150 nM bacterial AR (100 ng/well). Nonlabeled native and recombinant AR demonstrated an 85% maximal inhibition in these assays, confirming previous reports that AR has a relatively lower affinity than EGF to the EGFR.

Cell Lines and Cell Culture—The generation and characterization of the recombinant Ba/F3 cell lines used in this report have been described previously (17). These 11 cell lines, which express the four erbB family receptors singly and in pairwise combinations, are: LXSN/1 (vector control); EGFR/3; neu/12C; erbB-3/3; erbB-4/7; EGFR+neu/5D; EGFR+erbB-3/4A; EGFR+erbB-4/2A; neu+erbB-3/7A; neu+erbB-4/15A; and erbB-3+erbB-4/2B. Conditions for culturing these cell lines have been described earlier (17).

Stimulation and Analysis of ErbB Family Receptor Tyrosine Phosphorylation—The stimulation of erbB family receptor tyrosine phosphorylation in the recombinant Ba/F3 cell lines has been described previously (4, 17). Cells were stimulated with ligand at the following final concentrations: NRG, 94 ng/ml; EGF, TGF-α or HB-EGF, 100 ng/ml; AR, 1280 ng/ml. Dose-response experiments established that these hormone concentrations induced saturated levels of receptor tyrosine phosphorylation (data not shown).

Analysis of erbB family receptor tyrosine phosphorylation by immunoprecipitation and anti-phosphotyrosine immunoblotting has been described previously (4, 17). Immunoprecipitating anti-receptor antibodies were anti-EGFR mouse monoclonal antibody 528 (30), anti-Neu mouse monoclonal antibodies FSP16 (31), and TA-1 (Ab-5, Oncogene Science), anti-erbB-3 rabbit polyclonal antiserum SC-285 (Santa Cruz Biotechnology), and anti-erbB-4 rabbit polyclonal antiserum SC-283 (Santa Cruz Biotechnology). Specificity of anti-receptor antibodies has been verified by testing for cross-reactivity (data not shown) and previously we have established that hormone stimulation under these conditions does not appreciably modulate erbB family receptor protein levels (17).

RESULTS

Stimulation of Receptor Phosphorylation in Cell Lines Expressing a Single ErbB Family Receptor—In order to determine which receptors are activated individually by AR, TGF-α, and HB-EGF, we first evaluated the activation of erbB family receptor phosphorylation in the cell lines that express single erbB family receptors. Each of these factors stimulated the phosphorylation of EGFR, but not of neu or erbB-4 (Figs. 1*A* and *B*). In contrast, the positive control neuregulin-β (NRG) activated both neu and erbB-4 (Fig. 1*A*, *lane 12*; Fig. 1*B*, *lane 12*). All four EGF family peptides tested failed to stimulate erbB-3 tyrosine phosphorylation (Fig. 1*B*, *lanes 3–6*). This may be due to the minimal intrinsic kinase activity of erbB-3 (33, 34) and does not necessarily indicate that these hormones do not bind to erbB-3.

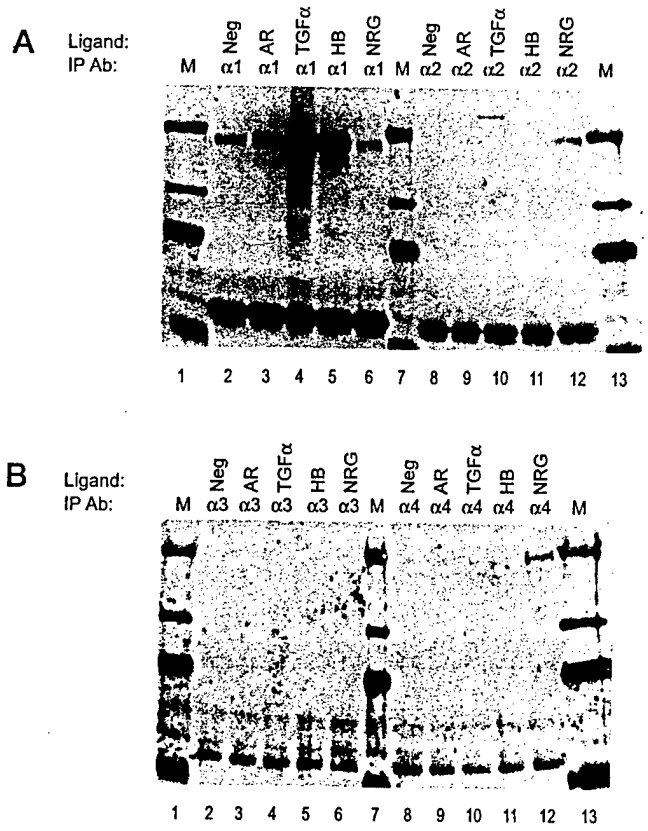

FIG. 1. **Comparison of receptor tyrosine phosphorylation in Ba/F3 derivatives expressing a single erbB family receptor and stimulated with AR, TGF-α, HB-EGF, or NRG.** Lysates from untreated or ligand-treated Ba/F3 derivatives were immunoprecipitated with anti-receptor antibodies and analyzed by immunoblotting with anti-phosphotyrosine antibody 4G10. Lysates from mock-stimulated cells are denoted *Neg*, lysates from AR-treated cells are denoted *AR*, lysates from TGF-α-treated cells are denoted *TGFα*, lysates from HB-EGF-treated cells are denoted *HB*, and lysates from NRG- treated cells are denoted *NRG*. Immunoprecipitating antibodies were *α1*, anti-EGFR; *α2*, anti-neu; *α3*, anti-erbB-3; *α4*, anti-erbB-4. *M* indicates prestained molecular mass markers (Bio-Rad), with mobilities comparable to 206, 117, 89, and 47 kDa. *A*, *lanes 2–6* are immunoprecipitates from the EGFR/3 cell line, and *lanes 8–12* are immunoprecipitates from the Neu/12C cell line. The neu tyrosine phosphorylation observed in response to NRG is apparently due to endogenous erbB-3 expression in Ba/F3 cells and is consistent with previous observations (17). *B*, *lanes 2–6* are immunoprecipitates from the ErbB-3/3 cell line, and *lanes 8–12* are immunoprecipitates from the ErbB-4/7 cell line.

Stimulation of Receptor Phosphorylation in Cell Lines Expressing Two Different ErbB Family Receptors—We then evaluated erbB family receptor phosphorylation in Ba/F3 cell lines that express pairwise combinations of receptors to assess the ability of these hormones to regulate erbB-2, erbB-3, and erbB-4 through transmodulation. AR, TGF-α, and HB-EGF stimulated EGFR receptor phosphorylation in the EGFR+neu, EGFR+erbB-3, and EGFR+erbB-4 cell lines (Figs. 2*A–C*). Furthermore, these hormones induced neu and erbB-4 tyrosine phosphorylation in the EGFR+neu and EGFR+erbB-4 cell lines, respectively (Figs. 2, *A* and *C*). In contrast, TGF-α activated erbB-3 in the EGFR+erbB-3 cell line, while AR and HB-EGF did not (Fig. 2*B*).

HB-EGF, TGF-α, and AR failed to activate erbB family re-

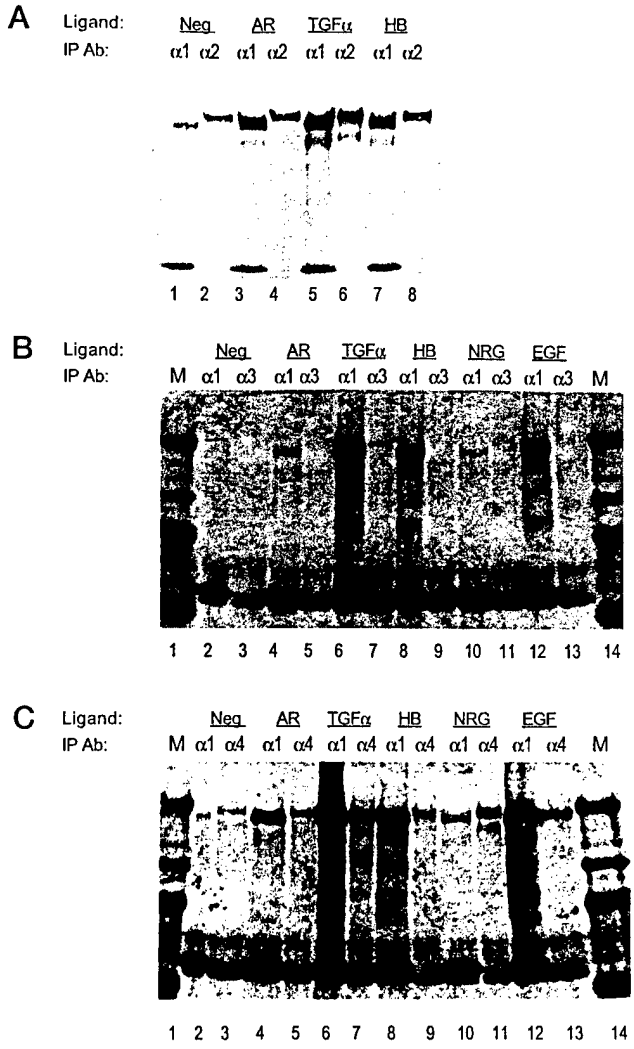

FIG. 2. **Comparison of receptor tyrosine phosphorylation in Ba/F3 derivatives expressing EGFR and another erbB family receptor and stimulated with AR, TGF-α, HB-EGF, NRG, or EGF.** Lysates from untreated or ligand-treated Ba/F3 derivatives were immunoprecipitated with anti-receptor antibodies and analyzed by immunoblotting with anti-phosphotyrosine antibody 4G10. Lysates from mock-stimulated cells are denoted *Neg*, lysates from AR-treated cells are denoted *AR*, lysates from TGF-α-treated cells are denoted *TGFα*, lysates from HB-EGF-treated cells are denoted *HB*, lysates from NRG-treated cells are denoted *NRG*, and lysates from EGF-stimulated cells are denoted *EGF*. Immunoprecipitating antibodies were *α1*, anti-EGFR; *α2*, anti-neu; *α3*, anti-erbB-3; *α4*, anti-erbB-4. *M* indicates prestained molecular mass markers (Bio-Rad), with mobilities comparable to 206, 117, 89, and 47 kDa. *A*, *lanes 1–8* are immunoprecipitates from the EGFR+neu cell line. *B*, *lanes 2–13* are immunoprecipitates from the EGFR+erbB-3 cell line. *C*, *lanes 2–13* are immunoprecipitates from the EGFR+erbB-4 cell line.

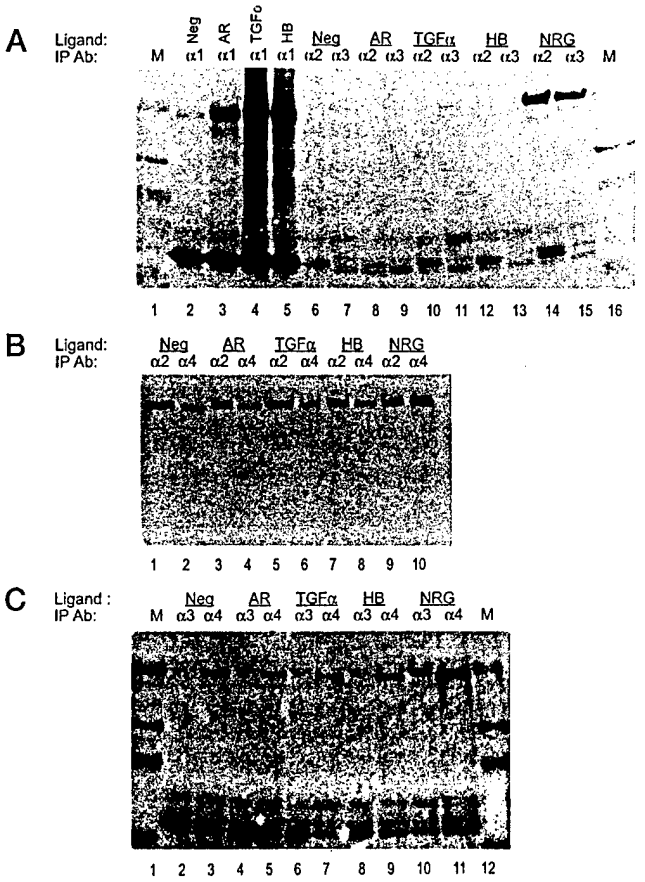

FIG. 3. **Comparison of receptor tyrosine phosphorylation in Ba/F3 derivatives expressing combinations of neu, erbB-3, or erbB-4 and stimulated with AR, TGF-α, HB-EGF, or NRG.** Lysates from untreated or ligand-treated Ba/F3 derivatives were immunoprecipitated with anti-receptor antibodies and analyzed by immunoblotting with anti-phosphotyrosine antibody 4G10. Lysates from mock-stimulated cells are denoted *Neg*, lysates from AR-treated cells are denoted *AR*, lysates from TGF-α-treated cells are denoted *TGFα*, lysates from HB-EGF-treated cells are denoted *HB*, lysates from NRG-treated cells are denoted *NRG*, and lysates from EGF-stimulated cells are denoted *EGF*. Immunoprecipitating antibodies were *α1*, anti-EGFR; *α2*, anti-neu; *α3*, anti-erbB-3; *α4*, anti-erbB-4. *M* indicates prestained molecular mass markers (Bio-Rad), with mobilities comparable to 206, 117, 89, and 47 kDa. *A*, *lanes 2–5* are immunoprecipitates from the positive control EGFR/3 cell line and *lanes 6–15* are immunoprecipitates from the Neu+erbB-3 cell line. *B*, *lanes 1–10* are immunoprecipitates from the Neu+erbB-4 cell line. The minimal stimulation of neu tyrosine phosphorylation by the positive control NRG in the neu+erbB-4 cell line is due to the high basal level of neu phosphorylation in this cell line and is consistent with previous observations (17). *C*, *lanes 2–11* are immunoprecipitates from the ErbB-3+erbB-4 cell line.

ceptors in the Neu+erbB-3, Neu+erbB-4, and ErbB-3+erbB-4 cell lines, even though NRG induced receptor phosphorylation in all three cell lines (Figs. 3*A–C*) and HB-EGF, TGF-α, and AR stimulated receptor phosphorylation in the EGFR cell line (Fig. 3*A*). Therefore, HB-EGF, TGF-α, and AR do not activate neu, erbB-3, or erbB-4 in the absence of EGFR expression and are presumably not ligands for these receptors.

Induction of IL-3-independent Survival or Proliferation in Ba/F3 Derivatives—While HB-EGF, TGF-α, and AR induced similar patterns of erbB receptor phosphorylation, another potential source of signaling specificity is that different ligands may couple the same erbB family receptors to distinct sets of cellular signaling pathways. To assess this possibility we tested whether HB-EGF, TGF-α, and AR induced different patterns of IL-3-independent survival or growth in the recombinant Ba/F3 cell lines. Our previous studies using these cell lines have established that activation of EGFR or neu, but not erbB-4, confers IL-3-independent survival, while activation of both EGFR and erbB-4 or EGFR and neu confers IL-3 independent proliferation (4, 17). As shown in Fig. 4, the vector control cell line failed to respond to any of the factors, while all four ligands induced IL-3-independent survival in the EGFR and EGFR+erbB-3 cell lines. (We judged that cultures exhibiting viable cell saturation densities of less than 10×10^3 cells/ml were nonresponsive to hormone, while cultures with viable cell saturation densities of $20–250 \times 10^3$ cells/ml were exhibiting survival but not proliferation and cultures with viable cell saturation densities of greater than 500×10^3 cells/ml were proliferating.) All four ligands stimulated IL-3-independent proliferation in the EGFR+neu and EGFR+erbB-4 cell lines.

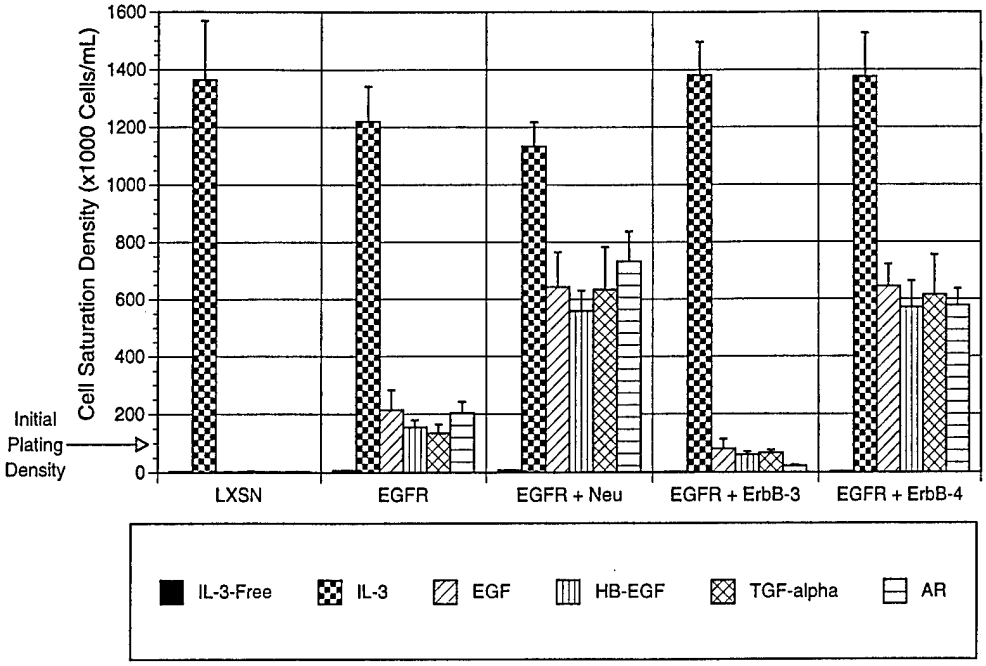

FIG. 4. **IL-3-independent saturation density of Ba/F3 cells stimulated with EGF, HB-EGF, TGF-α, or AR.** The stimulation and analysis of IL-3 independence in the recombinant Ba/F3 cell lines have been described previously (4, 17). The recombinant Ba/F3 cell lines were made quiescent by growth to saturation density and were seeded at a density of 100×10^3 cells/ml in medium lacking IL-3 (*IL-3-Free*), medium supplemented with IL-3, or in medium lacking IL-3 but supplemented with 10 ng/ml human recombinant EGF, 10 ng/ml HB-EGF, 10 ng/ml TGF-α, or 128 ng/ml AR. Cells were stained with trypan blue and counted in a hemacytometer daily 2–4 days after seeding to determine viable cell saturation densities. Each combination of cell lines and factors was tested 6–11 times and the arithmetic means of the viable cell saturation densities are indicated by the *filled bars*, while the standard errors of the means (32) are indicated by the *error bars*. Because cultures treated with IL-3-free medium exhibited viable saturation densities of less than 9×10^3 cells/ml, these values, represented by the *left-most bar* for each cell line, may not be apparent.

Consistent results were obtained in [^{3}H]thymidine incorporation assays of EGF and HB-EGF activity in the LXSN, EGFR, erbB-4, and EGFR+erbB-4 cell lines.[2] Therefore, in every cell line tested, EGF, HB-EGF, TGF-α, and AR induced growth responses that were quantitatively similar.

DISCUSSION

In this report we describe erbB family receptor tyrosine phosphorylation and coupling to cellular signaling pathways in response to the EGF family hormones AR, TGF-α, and HB-EGF. This study is the first analysis of the interactions of these hormones with the complete suite of erbB family receptors. In these assays TGF-α, AR, and HB-EGF were functionally nearly identical to each other and to EGF. These hormones stimulated only EGFR phosphorylation in the cell lines that express a single erbB family receptor, but TGF-α stimulated more EGFR phosphorylation than did HB-EGF or AR (Fig. 1). TGF-α, AR, and HB-EGF stimulated neu and erbB-4 phosphorylation in an EGFR-dependent manner (Fig. 2, *A* and *C*). However, while TGF-α stimulated erbB-3 phosphorylation in the EGFR+erbB-3 cell line, AR and HB-EGF failed to do so (Fig. 2*B*).

The reduced activity of AR relative to the other hormones has been observed in other experiments as well. EGF and TGF-α stimulate anchorage independent growth of NRK cells in the presence of transforming growth factor beta, while AR does not, and AR does not compete as well as EGF itself for ^{125}I-EGF binding to A431 cells (25). In our dose-response experiments, TGF-α stimulated markedly higher levels of EGFR phosphorylation than two independently prepared recombinant AR samples, not only in a recombinant Ba/F3 cell line, but also in ψ2 cells (an NIH/3T3-derived cell line) (35) engineered to overexpress EGFR.[3] Therefore, in our hands the reduced AR activity does not appear to be cell type- or preparation-specific, suggesting that AR may have reduced intrinsic activity compared to EGF and TGF-α. Nonetheless, AR might not be properly folded when expressed in *E. coli*, or the amino-terminal glycosylation that is present when AR is expressed in mammalian cells and is absent when AR is expressed in *E. coli* may modulate AR activity.

While we observed quantitative differences in receptor phosphorylation stimulated by TGF-α, AR, and HB-EGF, the physiologic responses to these factors were quantitatively quite similar. This was consistent with our earlier findings that the identity of receptors activated, rather than the number of activated receptors, determines the cellular response (4, 17). These factors all stimulated the IL-3-independent survival of EGFR cells and the IL-3-independent proliferation of EGFR+neu and EGFR+erbB-4 cells. In the sole exception, AR stimulated a weaker response in the EGFR+erbB-3 cell line compared to TGF-α or HB-EGF. This is concordant with the failure of AR and HB-EGF to stimulate erbB-3 phosphorylation in this cell line. These differences in TGF-α, AR, and HB-EGF activity suggests that there are threshold levels of activity required for the detection of erbB-3 phosphorylation and the efficient induction of IL-3-independent survival. Because EGFR expression in the EGFR+erbB-3 cell line is less than EGFR expression in the EGFR, EGFR+neu, and EGFR+erbB-4 cell lines (data not shown), the EGFR+erbB-3 cell line is likely to be more sensitive to the reduced activity of AR and HB-EGF.

The specification of biological responses to the EGF family/erbB receptor family signaling network is regulated at several distinct levels. We have previously demonstrated that the EGF

[2] K. Elenius and M. Klagsbrun, unpublished results.

[3] D. J. Riese II and D. F. Stern, unpublished results.

TABLE I

Regulation of erbB family receptor signaling and coupling to cellular responses by EGF family ligands

Results are abstracted from Riese *et al.* (4, 17) and Figs. 1–4 of this report.

Cell Line	Receptor	Group I EGF, AR, TGFα, HB-EGF		Group II NRGs		Group III BTC	
		Tyr phos[a]	Growth[b]	Tyr phos[a]	Growth[b]	Tyr phos[a]	Growth[b]
EGFR		+	S	−	N	+	S
Neu		−	N	+[c]	S[c]	−	N
ErbB-3		−	NT[d]	−	N	−	N
ErbB-4		−	NT[d]	+	N	+	N
EGFR+Neu	EGFR	+	P	*[c]	S[c]	+	P
	Neu	+		*[c]		+	
EGFR+erbB-3	EGFR	+	S	+	N	+	S
	ErbB-3	+		+		+	
EGFR+erbB-4	EGFR	+	P	+	P	+	P
	erbB-4	+		+		+	
Neu+erbB-3	Neu	−	NT[d]	+	S	−	N
	ErbB-3	−		+		−	
Neu+erbB-4	Neu	−	N[e]	+	S	+	S
	ErbB-4	−		+		+	
erbB-3+erbB-4	ErbB-3	−	N[e]	+	N	−	N
	ErbB-4	−		+		+	

[a] No increase in receptor tyrosine phosphorylation (Tyr phos) is indicated by "−," increased receptor tyrosine phosphorylation is indicated by "+," and ambiguity due to high basal levels of receptor phosphorylation is indicated by "*."

[b] The absence of an IL-3-independent response is indicated by "N," stimulation of IL-3 independent survival is indicated by "S," and stimulation of IL-3 independent proliferation is indicated by "P." "NT" indicates not tested.

[c] The responses to NRGs are apparently due to interactions with the endogenous erbB-3 in Ba/F3 cells (17).

[d] Given the absence of receptor tyrosine phosphorylation, no IL-3 independent response is expected.

[e] Results for EGF only. AR, TGF-α, and HB-EGF were not tested, but a negative response to these ligands identical to that observed for EGF is anticipated because none of these hormones activated receptor phosphorylation in these cell lines.

family of hormones can be divided into three groups according to their ability to stimulate phosphorylation of different erbB family receptors. The data presented here permit us to assign TGF-α, AR, and HB-EGF into these three functional groups (Table I). Group I of EGF family hormones now consists of EGF, TGF-α, AR, and HB-EGF. These peptides activate the EGFR in the absence of additional erbB family receptor expression and can stimulate the tyrosine phosphorylation of neu, erbB-4, and in some cases, erbB-3, in an EGFR-dependent manner. Group II of EGF family hormones consists of the NRGs. These peptides activate erbB-4 in the absence of additional erbB family receptor expression. However, the NRGs also bind to erbB-3, and activate it when coexpressed with any other erbB family receptor, and can stimulate neu or EGFR phosphorylation when these receptors are coexpressed with either erbB-3 or erbB-4. Group III of EGF family hormones consists solely of BTC. BTC activates EGFR or erbB-4 in the absence of additional erbB family receptor expression. Moreover, BTC stimulates neu tyrosine phosphorylation when neu is coexpressed with EGFR or erbB-4, and activates erbB-3 when erbB-3 is coexpressed with EGFR, but not when erbB-3 is coexpressed with erbB-4. Consequently, with one exception, hormonal activation of a single erbB family receptor can transmodulate any of the other three erbB family receptors. In different cells distinct EGF family hormones activate discrete sets of erbB family receptors, and biological responses to these hormones are specified in part by which erbB family receptors are expressed.

A corollary is that the activation of different erbB family receptors results in distinct biological responses (34, 36–46). We have observed such distinctions in our system. While EGFR activation results in the IL-3-independent survival of Ba/F3 cells and erbB-4 activation does not result in an IL-3-independent response, activation of both EGFR and erbB-4 results in IL-3-independent proliferation. Similarly, while activation of EGFR or neu alone results in IL-3-independent survival, activation of both EGFR and neu results in IL-3-independent proliferation (Table I). EGFR+erbB-4 cells stimulated with EGF, NRG, or BTC all undergo IL-3-independent proliferation, suggesting that the mechanism by which the receptors are activated does not appear to specify distinct biological responses. The stimulation of distinct biological responses by the four erbB family receptors apparently occurs through coupling of different sets of downstream signaling proteins to each receptor. Distinct sets of tyrosine phosphorylated proteins are coimmunoprecipitated with activated EGFR or activated erbB-4 (4). Experiments to identify these phosphoproteins and to determine their functional role in mediating erbB family receptor signaling are underway.

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Heregulin Induces *in Vivo* Proliferation and Differentiation of Mammary Epithelium into Secretory Lobuloalveoli[1]

Frank E. Jones, D. Joseph Jerry, Brad C. Guarino, Glenn C. Andrews, and David F. Stern[2]

Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06520-8023 [F. E. J., D. F. S.]; Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, Massachusetts 01003-6410 [D. J. J.]; and Pfizer Central Research, Groton, Connecticut 06340 [B. C. G., G. C. A.]

Abstract

Mammary gland development and differentiation is mediated through the combined activities of systemic hormones and locally synthesized growth factors. To determine the *in vivo* response of mammary epithelium to heregulin (HRG), we implanted Elvax pellets containing HRGα or HRGβ within the mammary glands of prepubescent female mice in the presence or absence of exogenous estradiol and progesterone (E/P). Mice treated in the same way with transforming growth factor α (TGF-α) were included as a positive control. Each growth factor treatment induced epithelial ductal branching in the presence or absence of E/P. In the absence of E/P, HRGβ did not affect terminal end bud formation, mammary epithelium branching, or ductal migration. In contrast, TGF-α and HRGα induced ductal branching and HRGα induced ductal migration in the absence of E/P. The overall mammary response to growth factors was potentiated by the concomitant presence of E/P. In every case, the *in vivo* mammary epithelial responses to HRGα were more robust than TGF-α. Limited lobuloalveolar development was also observed in growth factor-treated mammary glands when E/P was present. Histological examination of growth factor-induced lobuloalveoli revealed secretory products within the lumen of HRGα and HRGβ lobuloalveoli. TGF-α-induced lobuloalveoli lacked similar secretory products.

Introduction

Mammary gland development is unusual in that the vast majority of growth and differentiation occurs postnatally. In the prepubescent mouse, mammary ductal structures emanating from the nipple terminate in large bulbous structures referred to as TEBs.[3] With the onset of puberty, steroid hormones function as potent mitogens of TEB. This rapidly dividing cell population is responsible for ductal growth and branching during expansion of the mammary gland. During pregnancy, an additional pronounced growth cycle results in increased ductal branching and lobuloalveolar development. The lobuloalveoli terminally differentiate into milk-producing structures, and the extensive lobuloalveoli completely fill the interductal spaces during lactation (1, 2). These developmental processes are regulated through a complex series of events requiring the activities of both intraglandular and systemic hormones/growth factors (3–5). The steroid hormones estrogen and progesterone are major players in these developmental processes. However, the exact mechanisms underlying steroid hormone growth effects are not known and may involve a combination of direct effects and/or stimulation of growth factors which in turn mediate mammary gland development in a juxtacrine or autocrine fashion. Indeed, estradiol stimulates mammary epithelial expression and/or secretion of several EGF family members (6–10), and these growth factors have several important functional roles during mammary gland development (4, 5, 11).

Normal breast tissue expresses several EGF family members including EGF (12), TGF-α (12–16), amphiregulin (16–18), cripto-1 (16–18), and HRG (19). In addition, mammary gland expression of all four EGFR family members identified to date (*e.g.*, EGFR, erbB-2/HER-2/neu, erbB-3, and erbB-4) has been reported (19–22). A substantial body of evidence suggests that the EGF family of growth factors and their cellular receptors play an important role in both normal and malignant mammary gland development (4, 5, 11, 23–27). Most recently, the function of HRG in mammary gland development has been investigated. In mammary tumor cells, HRGs appear to have a mitogenic effect (28–31) or induce differentiation of mammary epithelium with the synthesis of milk proteins (30, 32–34). Yang *et al.* (19) examined the affects of HRG on mammary gland morphogenesis. In whole-organ culture, HRG stimulates lobuloalveolar development and the production of milk proteins. A putative role for HRG in lobuloalveolar development and milk production is further supported by the following observations; HRGα is expressed within the mammary mesenchyme adjacent to lobuloalveolar structures, and HRGα expression is regulated during mammary gland development and is only expressed during pregnancy (19). Therefore, HRG appears to be a po-

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[2] To whom requests for reprints should be addressed. Phone: (203) 785-4832; Fax: (203) 785-7467; E-mail: stern@biomed.med.yale.edu.

[3] The abbreviations used are: TEB, terminal end bud; EGF, epidermal growth factor; EGFR, EGF receptor; HRG, heregulin; TGF-α, transforming growth factor α; E/P, estradiol and progesterone; RP-HPLC, reverse phase high pressure liquid chromatography.

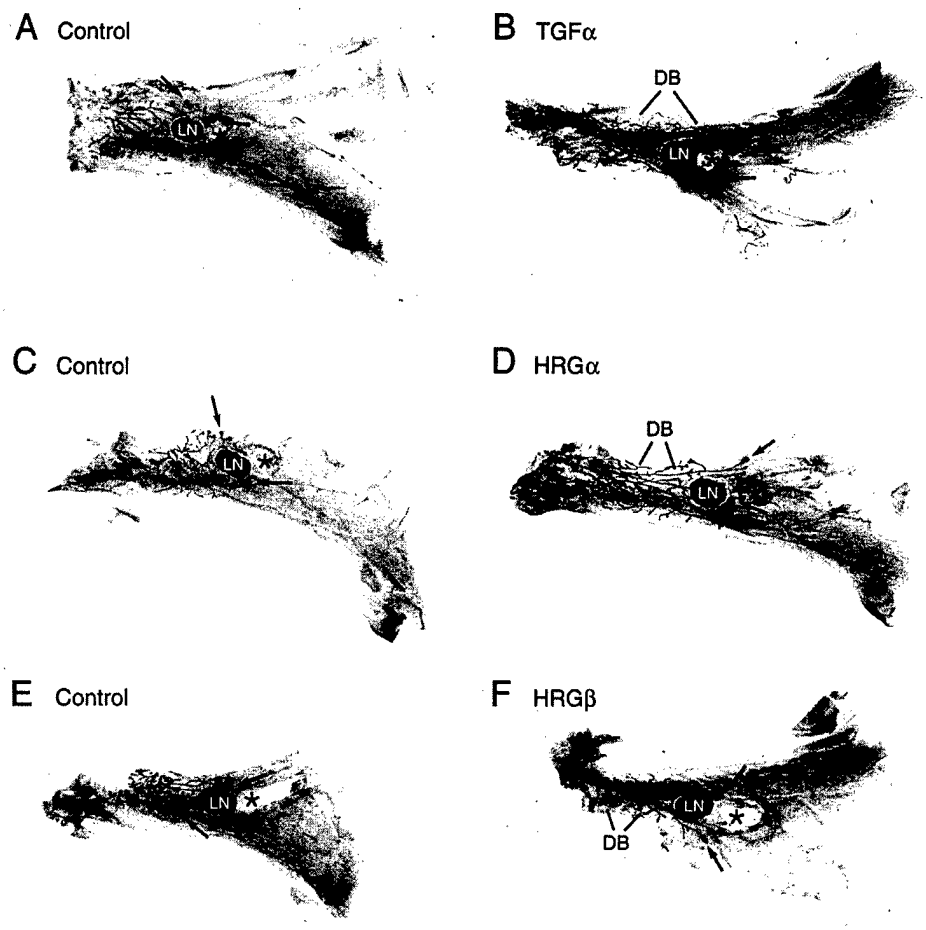

Fig. 1. Effect of growth factor treatment on mammary gland morphology in the absence of estradiol and progesterone. Control Elvax pellets and pellets containing growth factor were implanted within the surgically exposed number 4 inguinal mammary fat pad of 30-day-old female BALB/c mice. Implants were positioned (*) immediately anterior to the central lymph node (*LN*). Mice were sacrificed 3 days following implant insertion, and excised mammary glands were processed using the whole-mount procedure and stained with carmine (see "Materials and Methods"). Terminal end buds are indicated by *arrows,* and regions of growth factor-induced ductal branching (*DB*) are indicated. Implants contained 10 μg of TGF-α (*B*), 5 μg of HRGα (*D*), and 10 μg of HRGβ (*F*). Contralateral control for each sample is represented (*A, C,* and *E*).

tent and developmentally important mammary epithelial growth factor.

Despite these recent observations, the role of HRG in mammary gland morphogenesis *in vivo* has not been investigated. To address this issue, we have used a mouse model system to examine the direct effects of HRG on mammary epithelium under conditions similar to those where this growth factor normally functions. The mammary glands of prepubescent female mice were surgically implanted with Elvax pellets containing recombinant HRGα, HRGβ, or TGF-α in the presence or absence of steroid hormones. All three growth factors possessed differing levels of epithelial mitogenic activity *in vivo*. In addition, in the presence of steroid hormones, each growth factor induced epithelial differentiation into lobuloalveolar structures. However, only the HRG-treated lobuloalveoli underwent terminal differentiation, resulting in the lumenal accumulation of secretory products. Taken together, these experiments offer the first *in vivo* evidence for a role of HRG in mammary epithelial development and terminal differentiation into milk protein-secreting lobuloalveolar structures.

Results

HRG Induces Ductal Branching *in Situ.* HRG induces pleiotropic responses in cultured mammary epithelial cells (19, 28, 29, 31, 33–35); however, the *in vivo* response of mammary epithelium to this family of growth factors has not been investigated. As a first step toward identifying a biological role for HRG in mammary ductal morphogenesis, we surgically implanted slow-release pellets containing varying amounts of HRGα or HRGβ within the developing mammary fat pad of virgin female mice. HRGα and HRGβ are splice variants that possess differing EGF domains (36). Pellets lacking growth factor were inserted into the contralateral fat pad as a negative control. Previously, TGF-α has been shown to induce ductal branching and lobuloalveolar development in a similar experimental system (37) and was, therefore, included as a positive control in our experiments. The mice were sacrificed 3 days after implant insertion, and whole mounts of the mammary glands were examined for ductal morphogenesis and lobuloalveolar development.

When compared to contralateral controls, each growth factor induced ductal branching within the treated mammary gland (Figs. 1 and 2). Responses to growth factors in the

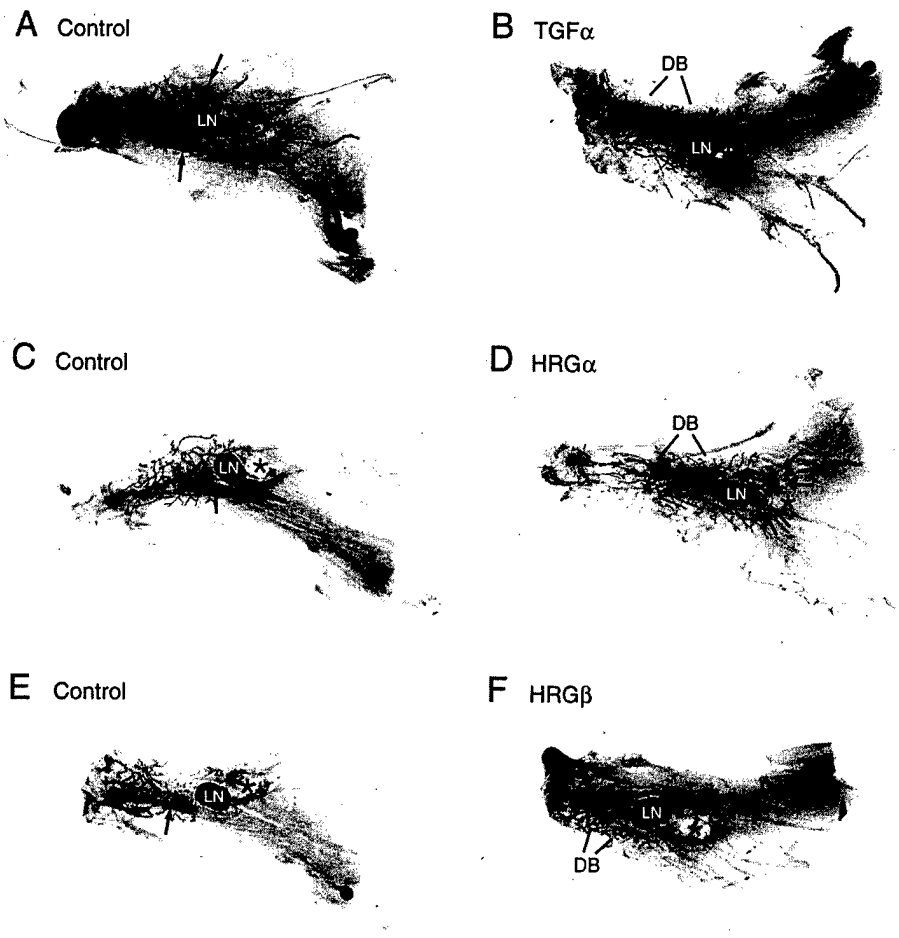

Fig. 2. Effect of growth factor treatment on mammary gland morphology in the presence of estradiol and progesterone. Control Elvax pellets containing 10 μg of 17 β-estradiol and 1 mg of progesterone (E/P) and pellets containing growth factor with E/P were implanted within the surgically exposed number 4 inguinal mammary fat pad of 30-day-old female BALB/c mice. Implants were positioned (*) immediately anterior to the central lymph node (*LN*). Mice were sacrificed 3 days following implant insertion, and excised mammary glands were processed using the whole-mount procedure and stained with carmine (see "Materials and Methods"). Terminal end buds are indicated by *arrows*, and regions of growth factor-induced ductal branching (*DB*) are indicated. Implants contained 10 μg of TGF-α (*B*), 5 μg of HRGα (*D*), and 10 μg of HRGβ (*F*). Paired contralateral control for each sample is represented (*A, C,* and *E*).

presence or absence of E/P were maximal with pellets containing 10 μg of TGF-α or HRGβ or 5 μg of HRGα (data not shown). These growth factor concentrations were used in all subsequent experiments. In the absence of E/P, each growth factor induced ductal branching in the region posterior to the central lymph node (Fig. 1, compare *panels A* to *B*, *C* to *D*, and *E* to *F*). However, differences between each growth factor could be identified. For example, the ductal branching observed in HRGβ-treated glands (Fig. 1*F*) was less extensive than glands treated with either TGF-α (Fig. 1*B*) or HRGα (Fig. 1*D*). In addition, where TGF-α and HRGβ treatment appeared to inhibit TEB formation (Fig. 1, *B* and *F*, respectively), HRGα not only induced TEB proliferation but also increased ductal migration anterior to the central lymph node (Fig. 1*D*).

Treatment of control mammary glands with E/P alone resulted in a slight increase in ductal diameter (compare Fig. 1*A* to Fig. 2*A*). Moreover, the mammary response to growth factors was potentiated by the presence of E/P because ductal branching induced by each growth factor was more pronounced in the presence of E/P (compare Fig. 1, *B, D,* and *F,* to Fig. 2, *B, D,* and *F,* respectively). The mammary epithelial responses to implants containing TGF-α (Fig. 2*B*) and HRGβ (Fig. 2*F*) were similar because both growth factors inhibited TEB formation. In contrast, HRGα induced TEB formation, and the overall epithelial response to HRGα was more robust (Fig. 2*D*) than either TGF-α (Fig. 2*B*) or HRGβ (Fig. 2*F*).

The extent of ductal branching, ductal growth, and TEB formation induced by each growth factor in the presence and absence of E/P was quantitated. Data from 10 mice, for each experimental condition, was subjected to statistical analysis. Due to high variability among mice, each quantitated parameter was normalized to the contralateral control within an individual animal. Although each growth factor induced ductal branching (Figs. 1 and 2), branching induced by HRGβ was statistically significant only in the presence of E/P (Fig. 3). In general, HRGα appeared to induce a more robust and pleiotropic response within treated mammary glands than either TGF-α or HRGβ. Indeed, ductal branching was more extensive in HRGα-treated glands whether in the presence or absence of E/P (Fig. 3). Moreover, whereas TGF-α and HRGβ appeared to slightly inhibit or had no affect on TEB formation, HRGα induced TEB proliferation in the presence of E/P (Fig. 3). Moreover, HRGα was the only growth factor to significantly increase ductal length within treated mammary glands

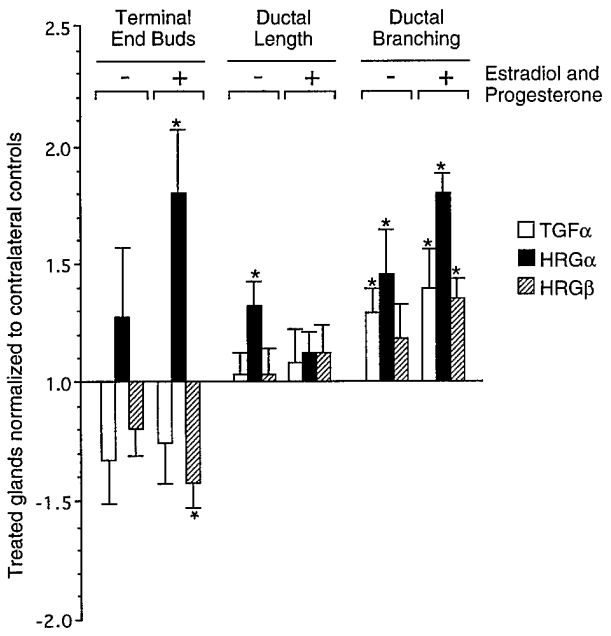

Fig. 3. Effects of growth factors on mammary epithelium. Ten 30-day-old female BALB/c mice were implanted with growth factor pellets at the growth factor's concentration of maximal response in the absence (−) or presence (+) of 10 μg of estradiol and 1 mg of progesterone. Mice were sacrificed 3 days following implant insertion, and excised mammary glands were processed using the whole-mount procedure and stained with carmine (see "Materials and Methods"). The number of TEBs, the ductal length of the longest duct per fat pad, and the number of ductal branches was determined for each treated and contralateral control gland. Treated glands were normalized to their contralateral controls, and the means plus 1 SD (*bars*) are represented above. Growth factor-induced phenotypes significantly different from contralateral controls (paired student *t* test; $P < 0.01$) are indicated (*). Implants contained 10 μg of TGF-α, 5 μg of HRGα, and 10 μg of HRGβ.

(Fig. 3). With the exception of increased ductal length induced by HRGα in the absence of E/P, the concomitant presence of E/P appeared to potentiate the mammary epithelial response to each growth factor tested (Fig. 3).

HRG Induces Mammary Lobuloalveolar Development and the Accumulation of Lumenal Secretory Products *in Vivo*. Whole mounts prepared from growth factor-treated mammary glands revealed extensive epithelial ductal branching. We next examined the terminal ducts within treated and control mammary glands for evidence of lobuloalveolar structures. As expected, untreated glands or glands treated with E/P alone did not develop lobuloalveolar structures. In contrast, a limited extent of lobuloalveolar development was observed in a majority of mammary glands treated with growth factor (Fig. 4, *A-C*). Growth factor-induced lobuloalveoli required the concomitant presence of E/P because similar structures were not observed in glands treated with growth factors alone. Consistent with previous results, the effect of HRGα was more robust than either TGF-α or HRGβ. HRGα induced lobuloalveolar development in 77% of treated mammary glands, whereas TGF-α and HRGβ induced lobuloalveoli in 39 and 46% of treated glands, respectively (13 glands were examined for each treatment). Histological examination of growth factor-induced lobuloalveoli revealed numerous epithelial buds typical of these structures (Fig. 4, *D-F, arrows*). In addition, HRGα- and HRGβ-induced lobuloalveoli exhibited accumulation of lumenal secretory products (Fig. 4, *E* and *F*), which stained positive for β-casein by immunohistochemistry (data not shown). Similar accumulations were not observed in TGF-α-induced lobuloalveoli (Fig. 4*D*).

Discussion

Mammary gland development involves a complex and highly regulated sequence of postnatal events. Recently, expression of an EGF-related subfamily of growth factors termed the neu differentiation factors or HRGs was detected *in vivo* within connective tissue juxtaposed to fully differentiated, milk-secreting lobuloalveoli (19). To determine if HRG plays a role in mammary epithelial growth and/or differentiation *in vivo*, we inserted slow-release pellets containing HRG within mammary glands of prepubescent mice and analyzed the *in vivo* response of mammary epithelium to these growth factors. We found that HRGα and HRGβ induced epithelial branching and differentiation into lobuloalveolar structures, as does a related growth factor, TGF-α. However, histological examination of TGF-α- and HRG-induced lobuloalveoli revealed a striking difference; HRGα and HRGβ stimulated the accumulation of lumenal secretory products, including the milk protein β-casein, within treated lobuloalveoli. TGF-α-induced lobuloalveoli lacked similar lumenal accumulations. These results suggest that HRG can induce terminal differentiation of mammary epithelium *in vivo* into milk protein-secreting lobuloalveolar structures.

The epithelial response to growth factor implants was potentiated by the concomitant presence of estradiol and progesterone. Indeed, lobuloalveoli were only observed in the presence of these steroid hormones. A similar requirement of estradiol and progesterone for EGF- and HRG-induced lobuloalveoli in mammary organ culture has been reported (4, 19, 38). Some evidence suggests that the requirement of exogenous steroid hormones may also reflect strain differences. For example, TGF-α implants induce lobuloalveoli development in CH3/HeN mice in the absence of exogenous estradiol and progesterone (37). In contrast, our results indicate that induction of lobuloalveoli by TGF-α or HRG in BALB/c mice requires the concomitant presence of exogenous estradiol and progesterone. Although we did not perform experiments to determine if estradiol or progesterone alone could augment mammary responses to growth factors, substantial evidence indicates that both estradiol and progesterone have independent proliferative effects on mammary epithelium. Furthermore, co-administration of these hormones enhances independent proliferative effects (3). Using mice carrying a null mutation in the progesterone receptor, Lydon *et al.* (39) demonstrated the *in vivo* requirement of progesterone in ductal epithelium proliferation and lobuloalveoli differentiation. Thus, it seems probable that progesterone contributes to the growth factor-induced lobuloalveolar development observed in our experiments. Although estradiol is considered to be the steroid hormone most directly involved in mammary epithelial proliferation (3), the exact role of estradiol in mammary development is poorly defined. The

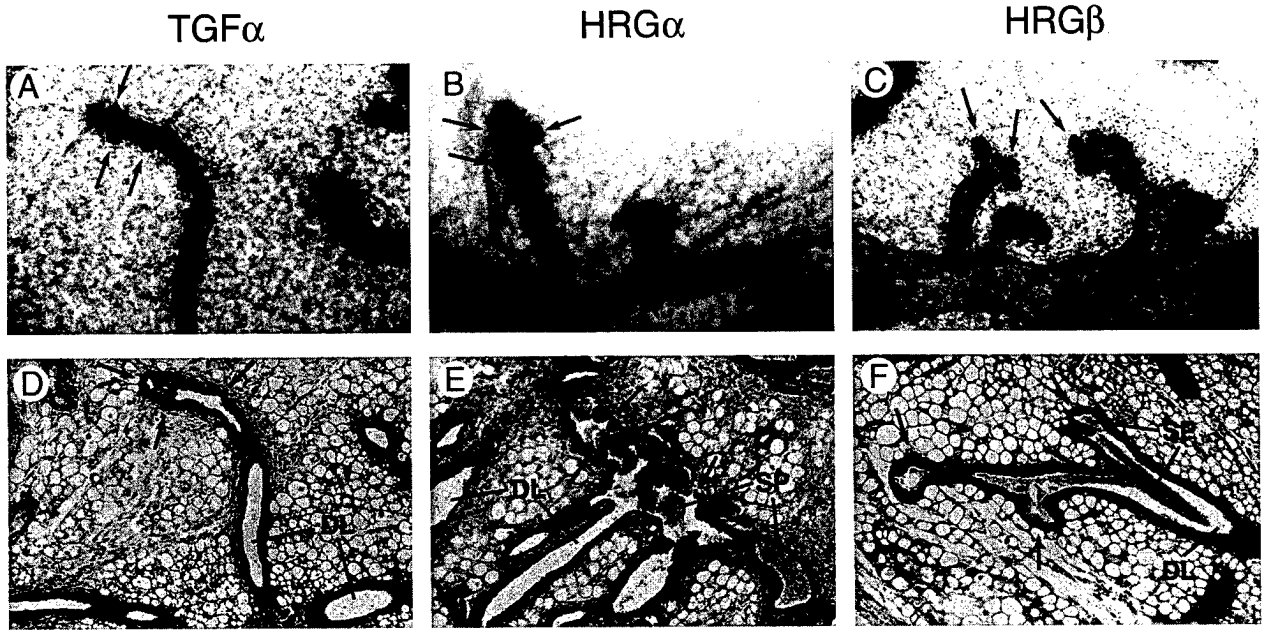

Fig. 4. Growth factor-induced lobuloalveolar development in prepubescent female mice. Elvax pellets containing growth factor with 10 μg of 17 β-estradiol and 1 mg of progesterone were implanted within the surgically exposed number 4 inguinal mammary fat pad of 30-day-old female BALB/c mice. Mice were sacrificed 3 days following implant insertion, and excised mammary glands were processed using the whole-mount procedure and stained with hematoxylin (see "Materials and Methods"). Photomicrographs of paraffin sections revealed lobuloalveolar structures in growth factor-treated glands (*A-C*). *Arrows* indicate epithelial buds typical of these structures. Histological examination of paraffin-embedded lobuloalveoli stained with H&E (*D* and *E*) revealed secretory products (*SP*) within the ductal lumens (*DL*) of HRGα- and HRGβ-treated mammary glands (*E* and *F*, respectively). Implants contained 10 μg of TGF-α (*A* and *D*), 5 μg of HRGα (*B* and *E*), and 10 μg of HRGβ (*C* and *F*).

reason for this lack of clarity lies in the fact that in addition to independent proliferative effects, estradiol also induces mammary expression of several growth factors, including the EGF family members EGF (7), TGF-α (6, 7, 40), and amphiregulin (9, 41). Mammary gland expression of these EGF family members may directly affect mammary development and thereby augment the epithelial response to growth factor implants observed in our experiments.

The qualitative and quantitative differences in mammary epithelial responses to TGF-α and HRG can be explained through functional differences of the two growth factors. Histochemical analysis of mouse mammary glands reported elsewhere revealed dramatic differences in the cellular localization and expression of these growth factors. Mammary gland expression of TGF-α was detected during each epithelial developmental stage with the exception of lactation, and immunostaining within the cap cell layer of the TEB and epithelial cells of subtending ducts was observed (12). In contrast, expression of HRG is induced during pregnancy within the connective tissue adjacent to ductal and lobuloalveolar structures (19). Differing mammary epithelial responses to TGF-α and HRG may also reflect the activation of different signaling tyrosine receptor kinases within these cellular populations. TGF-α binds directly to the EGFR (26) and can activate erbB-2 (42), erbB-3, and erbB-4,[4] presumably through a ligand-driven receptor cross-phosphorylation mechanism (43, 44) also referred to as "transmodulation" (24, 45). Similarly, HRG binds directly to erbB-3 (46, 47) and erbB-4 (47) and can drive the activation of EGFR and erbB-2 (46–48). Evidence from *in vitro* experiments indicates that cellular responses to signaling by this family of receptors can be radically different, depending upon both the transmodulation partner and the activating growth factor (48–51). Therefore, one prediction follows that signaling by EGFR family members *in vivo* would also induce a diversity of cellular responses that are dependent upon the activating growth factor. Cellular responses to HRG *in vivo* appear to be regulated primarily but not exclusively through erbB-2 signaling. Disruption of HRG or erbB-2 in transgenic mice results in a similar embryonic lethal phenotype characterized by nearly identical heart malformations and neural crest development defects (52, 53). Moreover, expression patterns within the developing rhombencephalon suggest that a HRG: erbB-2 autocrine or paracrine signaling relationship has been disrupted in these mice (52, 53). These observations further support a direct relationship between HRG and erbB-2 signaling. A similar relationship may mediate HRG activity in mammary epithelium, and we are presently designing experiments to examine this possibility.

In our experiments, the *in vivo* response of mammary epithelium to HRGα was more robust than HRGβ. This result was surprising because *in vitro* experiments consistently identify HRGβ as the more potent growth factor (28, 30, 31, 33). However, we used chemically synthesized and bacterial recombinant peptides in our experiments, which may not

[4] D. J. Riese II, E. Kim, G. Allison, S. Buckley, M. Klagsbrun, G. D. Plowman, and D. F. Stern. J. Biol. Chem., in press.

represent the complete activities of full-length HRG protein. Alternatively, the enhanced mammary response to HRGα may reflect a physiological role for HRGα and not HRGβ in mammary gland development. Indeed, only HRGα isoforms are expressed in the mammary gland, and this expression is induced during pregnancy (19). Thus, the HRGα expression pattern strongly correlates with the *in vivo* function identified in this communication. Our experiments provide the first demonstration of an important *in vivo* role for HRG in mammary epithelium proliferation and differentiation into secretory lobuloalveoli. In conclusion, we propose that HRGα is the physiologically relevant HRG isoform expressed within the developing mammary gland, and HRGα plays an important role in the differentiation of mammary epithelium into milk-secreting lobuloalveoli.

Materials and Methods

Plasmid Construction. The human HRG β1 cDNA fragment corresponding to residues 177–244 (54) was subcloned into the pNB261 bacterial expression vector as follows. Poly(A) mRNA was isolated from cultured human MDAMB231 cells (American Type Culture Collection) by use of the Fast Track mRNA isolation kit (InVitrogen), according to the manufacturer's instructions. The HRG β1 cDNA fragment corresponding to residues 177–244 was amplified by a 30-cycle reverse transcription-PCR procedure using the RNA Gene Amp kit (Perkin-Elmer Corp.) and the primers incorporating 5′ *Eco*RI (sense 5′-CGCGAATTCTATGAGCCATCT-TGTAAAATGTGC) and *Hind*III (anti-sense 5′-CGCGAAGCTTAGTA-CAGCTCCTCCGCCTCCAT) linkers. The 204-bp amplified fragment was digested with *Eco*RI/*Hind*III and inserted into the same sites of the Bluescript vector pCRII (Stratagene). The nucleotide sequence of the 204-bp insert was confirmed by use of an Applied Biosystems Automated Sequencer using standard methods. The sequenced 204-bp insert was excised from pCRII by digestion with *Eco*RI/*Hind*III and subcloned downstream of the trp-inducible promoter using the same restriction sites of the pNB261 expression vector (construct pHerβ1ST). The sequence of the 204-bp human HRG β1 insert was confirmed as described above.

Expression and Purification of Human Recombinant HRG β1 (177–244). For large scale fermentation and expression of HRG β1 (177–244), pHERβ1ST was transformed into the *Escherichia coli* strain GE81. Bacterial cells from a 10-liter fermentation in modified M9 medium (55) were harvested by centrifugation, and expression was induced by resuspending the bacteria pellet into fresh modified M9 medium lacking tryptophan. After an induction period of 4 h, a total of 69 g of cell paste was recovered by centrifugation. Expression of the predicted 7000-Da product peaked at 3 h postinduction. A 25-g bacterial pellet was resuspended into 50 ml of lysis buffer [20 mM Tris (pH 8.0), 40 mM NaCl, 0.25 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride] and passed through a French press twice. The lysate was centrifuged for 45 min at 12,000 rpm, and the pellet was resuspended into 20 ml of lysis buffer. Fifteen mg of hen egg white lysozyme (Sigma Chemical Co.) was added, and the mixture was incubated at room temperature for 20 min. Three hundred μl of 1 mg/ml DNase and 800 μl of 100 mM $MgCl_2$ were added, and the mixture was incubated at room temperature for an additional 15 min. The suspension was centrifuged for 15 min at 12,000 rpm, and the final inclusion body pellet was washed twice with 1.0% NP40 and once with ddH_2O and lyophilized to dryness to yield 500 mg of dried inclusion bodies. A suspension of 10 mg of inclusion bodies in 2.0 ml of 50 mM Tris (pH 6.0), 6 M guanidinium hydrochloride, and 200 mM DTT was heated at 37°C for 2 h and filtered through a Costar 3.5-μm spin-filter; then the filtrate was diluted into 100 ml of folding buffer [50 mM Tris (pH 8.6), 1 M urea, 1.5 mM glutathione, 0.75 mM glutathiol, and 10 mM methionine] and stirred for 5 days at 4°C. The folded, oxidized protein was isolated by RP-HPLC on a VYDAC C-4 reverse phase column using an acetonitrile/ddH_2O/0.1% trifluoroacetic acid gradient. The isolated protein was homogeneous by RP-HPLC and capillary electrophoresis, and was composed of 206 μg by amino acid analysis. The protein exhibited a mass of 7877.8 Da by electrospray mass spectrometry [theoretical mass for oxidized HRG β1 (177–244) is 7878.1 Da].

Growth Factors. HRGα 177–228 (HRGα 52) was synthesized on an Applied Biosystems 430A peptide synthesizer using standard *tert*-butyloxycarbonyl chemistry protocols provided by the manufacturer (version 1.40; *N*-methylpyrolidone/hydroxybenzotriazole). Peptide was purified by RP-HPLC, characterized by electrospray mass spectroscopy, and analyzed for disulfide bonding as described previously (56). Peptide quantities were determined by amino acid analysis. Human recombinant TGF-α was purchased from Collaborative Biomedical Products.

Implant Preparation. Growth factor peptides and steroid hormones were encapsulated within Elvax pellets essentially as described elsewhere (57). Briefly, a lyophilized mixture containing growth factor peptide and, where indicated, the steroid hormones 17-β-estradiol (10 μg; Sigma) and progesterone (1 mg; Sigma) was suspended in 25 μl of Elvax (generously donated by Elf Atochem, Philadelphia, PA) dissolved previously in dichloromethane (15% w/v). The entire suspension was transferred to an Eppendorf tube, snap-frozen in liquid nitrogen, and dried under vacuum. The dried Elvax pellet was compressed between tweezers such that the final pellet was ~1 mm in diameter and weighed 2–3 mg.

Surgical Implantation. Thirty-day-old virgin female BALB/c mice (Charles River) were used in all experiments. Mice were anethesized with an i.p. injection of 250–350 μl of avertin [20 mg/ml 2,2,2-tribromoethanol (Aldrich) in saline]. The number 4 inguinal mammary fat pad was surgically exposed, and a 2-mm incision was made through the mammary fat pad outer membrane immediately anterior to the central lymph node. The Elvax pellet was placed within the incision and immobilized under the mammary fat pad outer membrane. Control pellets lacking growth factor were inserted into the contralateral number 4 inguinal mammary fat pad. The wounds were closed using surgical staples, and the mice were allowed to recover under a heat lamp.

To determine the response range and saturation point for each growth factor, mice were implanted with Elvax pellets containing 0.5, 1.0, 2.0, 5.0, 10, or 20 μg of growth factor. In another series of experiments, pellets contained 10 μg of 17 β-estradiol and 1 mg of progesterone (E/P) in addition to growth factor.

Whole-Mount Preparation of Mammary Gland. Mice were sacrificed 3 days following placement of implants. The entire number 4 inguinal mammary fat pad was removed at the nipple and spread onto a precleaned glass slide. The fat pad was air-dried for 10 min and fixed in acidic ethanol (75% ethanol and 25% acetic acid) for 1 h at room temperature. The tissue was incubated in 70% ethanol for 15 min and ddH_2O for 5 min. Ductal structures were stained in carmine solution [0.2% carmine and 0.5% aluminum potassium sulfate (both from Sigma)] for 12–16 h at room temperature. The stained tissue was dehydrated through graded ethanol, defatted in acetone, and cleared in toluene for 12–16 h. The stained and cleared mammary fat pad was mounted under coverslip with Permount (Fisher) and photographed with a slide duplicator.

Histological Examination. For histological examination of mammary gland ductal structures, fat pads were fixed in 4% paraformaldehyde, stained in hematoxylin or carmine solution, dehydrated through graded ethanol into xylene, and cleared in methyl salicylate (Sigma). Ductal structures identified under a dissecting microscope were excised, blocked in paraffin, sectioned at 4 μm, and stained with H&E using standard procedures.

Acknowledgments

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