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

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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

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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

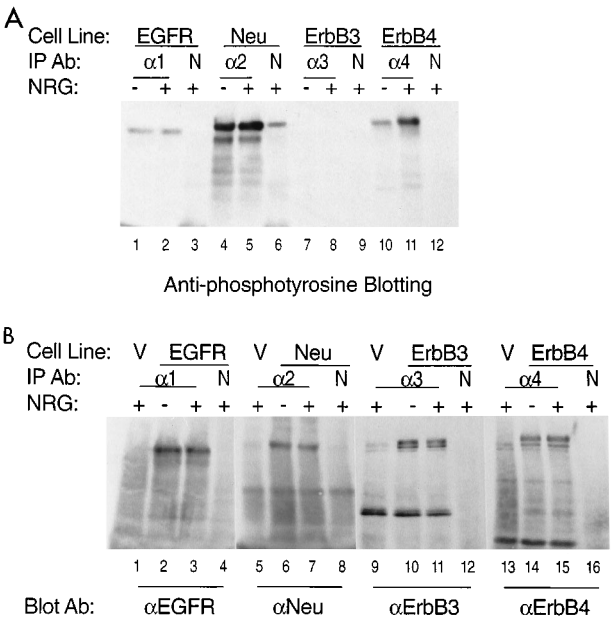

FIG. 1. Regulation of receptor tyrosine phosphorylation by NRG in single recombinant Ba/F3 derivatives. Untreated or NRG-stimulated cell lines were immunoprecipitated with antireceptor antibodies, and portions of immunoprecipitates were analyzed by immunoblotting with antiphosphotyrosine (A) or antireceptor (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.

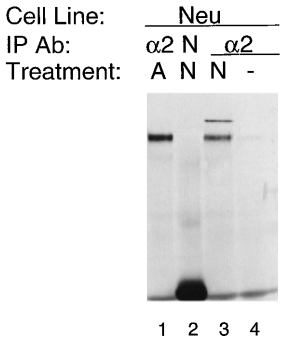

FIG. 2. Regulation of neu/12C cells by NRG. neu/12C cells were incubated with NRG dilution buffer (−), the agonistic anti-neu antibody TAb 250 (46) (A), or NRG (N). The immunoprecipitating antibody (IP Ab) used for immunoprecipitating lysates was anti-neu antibody (α2) or normal mouse serum (N); after 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

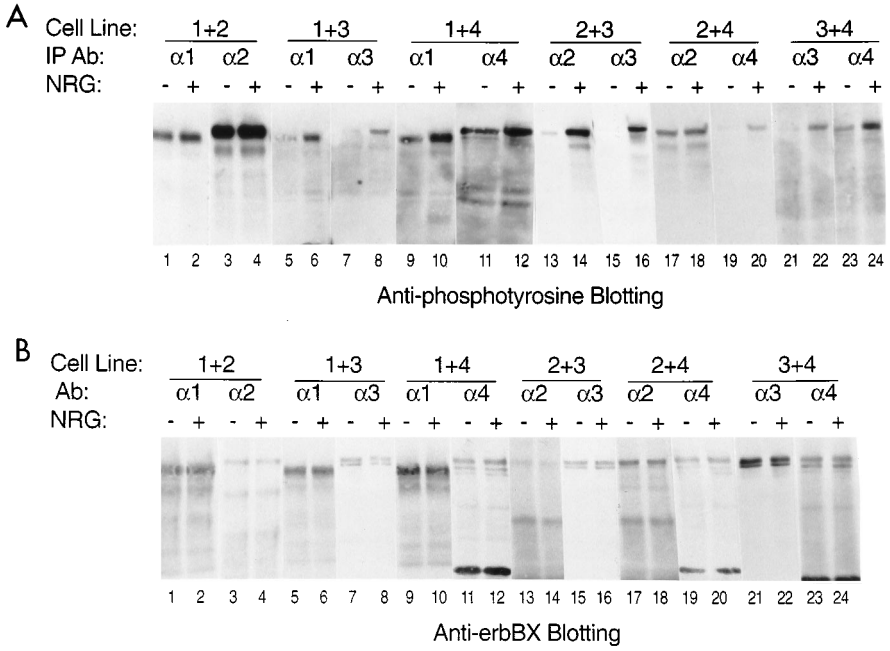

FIG. 3. Regulation of receptor tyrosine phosphorylation in double recombinant Ba/F3 derivatives. Untreated or NRG-treated cells were immunoprecipitated with antireceptor antibodies and analyzed by immunoblotting with antiphosphotyrosine (A) or antireceptor (B) antibodies. Cell lines: 1+2, EGFR + neu; 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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