A Differential Requirement for the COOH-terminal Region of the Epidermal Growth Factor (EGF) Receptor in Amphiregulin and EGF Mitogenic Signaling*

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The epidermal growth factor receptor (EGFR) mediates the actions of a family of bioactive peptides that include epidermal growth factor (EGF) and amphiregulin (AR). Here we have studied AR and EGF mitogenic signaling in EGF-receptor-devoid NR6 fibroblasts that ectopically express either wild type EGFR (WT) or a truncated EGFR that lacks the three major sites of autophosphorylation (c‘1000). COOH-terminal truncation of the EGFR significantly impairs the ability of AR to (i) stimulate DNA synthesis, (ii) elicit Elk-1 transactivation, and (iii) generate sustained enzymatic activation of mitogen-activated protein kinase. EGFR truncation had no significant effect on AR binding to receptor but did result in defective GRB2 adaptor function. In contrast, EGFR truncation did not impair AR mitogenic signaling, and in c‘1000 cells EGF was able to stimulate the association of ErbB2 with GRB2 and SHC. Elk-1 transactivation was monitored when either ErbB2 or a truncated dominant-negative ErbB2 mutant (ErbB2-(1–813)) was overexpressed in cells. Overexpression of full-length ErbB2 resulted in a strong constitutive transactivation of Elk-1 in c‘1000 only by slightly stimulated Elk-1 in WT or parental NR6 cells. Conversely, overexpression of ErbB2-(1–813) inhibited EGF-stimulated Elk-1 transactivation in c‘1000 but not in WT cells. Thus, the cytoplasmic tail of the EGFR plays a critical role in AR mitogenic signaling but is dispensable for EGF, since EGF-activated truncated EGFRs can signal through ErbB2.

The ErbB family of receptors that include the epidermal growth factor receptor (EGFR, ErbB1, HER1) (1), ErbB2 (HER2, neu) (2), ErbB3 (HER3) (3), and ErbB4 (HER4) (4) mediate the biological actions of a family of growth factors that are structurally related to EGF. Signaling from ErbBs involves a process of receptor homo- and heterodimerization that is initiated by specific engagement of ligand by the extracellular domain of one or more of the ErbB receptors (reviewed in Ref. 5). Within the family of EGF-like growth factors are a subset of bioactive peptides that have all been shown to bind to and activate the EGFR and include EGF (6, 7), transforming growth factor α (8, 9), amphiregulin (AR) (10–12), heparin-binding EGF-like growth factor (13), betacellulin (14), and epiregulin (15). In the case of AR and heparin-binding EGF cell-surface heparan sulfate proteoglycan (HSPG) is essential for ligand-induced activation of the EGFR (16–18). The biological purpose of the apparent redundancy in ligands that can activate the EGFR is not fully understood but may represent a mechanism of receptor activation and signaling that can be modulated by expression of the various ErbBs and HSPGs.

Like EGF (reviewed in Ref. 7), AR is a potent mitogen for fibroblasts (19), keratinocytes (19–21), and both normal and malignant epithelial cells (11, 16, 19, 22–27). AR is commonly overexpressed in human malignancies of the colon (22, 28), stomach (29–31), breast (32), and in the pancreas, overexpression correlates with reduced patient survival (26, 33). In vitro, AR has been shown to function in an autocrine manner to drive the proliferation of malignant cells of the colon (22), breast (24), cervix (34), prostate (35), and pancreas (26, 27). The EGFR is an 1186-amino acid residue protein that consists of an extracellular ligand binding domain, a transmembrane domain, an intracellular tyrosine kinase domain, and a COOH-terminal region that contains autophosphorylation sites at tyrosines 992, 1068, 1086, 1148, and 1173 (1, 36). The COOH-terminal tail of the EGFR is believed to regulate access of substrates to the catalytic domain and, when phosphorylated, to provide docking sites for Src homology 2 (SH2) domain-containing proteins (37, 38). Paradoxically, several studies have shown that this EGFR region is not required for EGF-stimulated mitogenesis or transformation (39–44) but is critical to the cell motility that occurs in response to EGF (40). With the exception of EGF, little is currently known regarding the structural requirements within the EGFR that are critical to signaling by the various EGF ligands. Previous work has demonstrated a requirement for EGFR kinase activity but not for COOH-terminal autophosphorylation sites in ligand-stimulated activation of signal transducers and activators of transcription (STAT) 1, 3, and 5 by either AR or EGF.
and no significant differences were observed between the ligands (45). In contrast to those findings we report here that the COOH-terminal region of the EGFR is critical for AR-induced mitogenic signaling, whereas it appears to be expendable for EGF-mediated mitogenesis. The purpose of this work was to identify the molecular basis for this difference. The results revealed that this differential requirement for the cytoplasmic tail of the EGFR in AR and EGF signaling can be attributed to ErbB2.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Either human wild type (WT) or truncated EGFR corresponding to the amino-terminal 1000 residues (c’1000) was expressed in EGFR-devoid NR6 fibroblasts (46) via retrovirus-mediated transduction (39, 40). These polyclonal cell lines have been shown to express comparable levels of EGFR and bind EGF with very similar affinities (40). The cells were maintained in culture as described previously (40).

**Antibodies, Reagents, and cDNAs**—Antibodies to the EGFR (Ab-5) and ErbB2 (c-neu, Ab-3) were obtained from Oncogene Science (Uniondale, NY), and biotinylated PY-20 antibody was purchased from ICN Biomedical, Inc. (Costa Mesa, CA). The human-specific antibody against ErbB2 (c-neu) was obtained from NeoMarkers (Union City, CA), and antibodies to SHC, SHP-2 (PTP1D), mitogen-activated protein kinase (MAPK) (anti-pan ERK) were obtained from Transduction Laboratories (Lexington, KY). The biotinylated 4G10 and anti-EGFR L22 monoclonal antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Antibodies directed against the phosphorylated activated form of p44 mitogen-activated protein kinase were obtained from New England Biolabs (Beverly, MA). GRB2-glutathione S-transferase (GST) fusion protein were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The 87-amino acid residue form of recombinant human AR was generated as described previously (12). Recombinant human EGF was obtained from PeproTech, Inc. (Rocky Hill, NJ). The pFPR-Luc and pF2A-Erk-1 plasmids were obtained from Stratagene (La Jolla, CA). Human cDNA 3.1/His/agarose was purchased from Invitrogen (Carlsbad, CA). An XhoI fragment encoding full-length human ErbB2 cDNA was generously provided by Jacelyn Pierce (National Cancer Institute, National Institutes of Health) and subcloned into pcDNA 3.1 (Invitrogen). The cDNA encoding ErbB2 (1–813) was generated by digesting the full-length ErbB2 construct with SacII and HindIII, blunting the ends with the large Klenow fragment of DNA polymerase I and ligating the ends of the plasmid. The sequence of this construct was confirmed by DNA sequencing.

**DNA Synthesis Assay**—Cells were grown to confluency in 96-well plates, serum-starved for 3 h, and treated with serum-free medium containing EGF or AR. After 6 h, the cells were pulsed for an additional 16 h with [3H]thymidine (2 mCi/well; Amersham Pharmacia Biotech). DNA was harvested, and the incorporation of [3H]thymidine into newly synthesized DNA was quantitated as described previously (23).

**ELK-1 Transactivation Assay**—Subconfluent cells in 6-well plates were incubated with 1 ml of Fx-6 transfection lipid (Invitrogen) in Opti-Mem (Life Technologies, Inc.) containing 0.5 µg of pFR-Luc, 50 ng of pF2A-Erk-1, and 100 ng of pcDNA 3.1/His/agarose. The proteins were added to 4 ml of 10% FBS DMEM. After 4 h at 37°C, the cells were plated in 1 ml of medium containing EGF or AR. After 6 h, the cells were washed four times with ice-cold PBS, harvested, and subjected to luciferase assay (Promega). A total of 250 µl of cell extract was used, and the luciferase activity was measured using a Turner Design luminometer.

**Western Blotting**—Proteins were separated on SDS-PAGE gels and transferred to nitrocellulose membranes (Novex). Membranes were probed with 0.3 µg/ml primary antibody, and detection was performed using the Vectastain ABC Elite Kit (Vector Laboratories) and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

**RESULTS**

**Characterization of NR6 Cells Expressing Wild Type or c’1000 EGFRs**—Either wild type (WT) or truncated EGFR lacking the COOH-terminal 186 amino acid residues (c’1000) was expressed in EGFR-devoid NR6 fibroblasts (46) via retrovirus-mediated transduction (40). The WT and c’1000 polyclonal cell lines possess comparable numbers of EGF-binding sites with similar affinities as described previously (40). Both WT and c’1000 receptors undergo ligand-dependent endocytosis with rates of 0.080 and 0.042 pm per min, respectively.2 WT and c’1000 cells express detectable levels of murine ErbB2 (see below) but do not respond to heregulin α or β indicating that these cells do not express significant quantities of the EGF-related receptors, ErbB3 or ErbB4.3 WT and c’1000 cell lines express equivalent levels of GRB2, ErbB2, SHP-2, SHC, and ERK 2 as confirmed by Western blotting of whole cell lysates (data not shown).

**The COOH-Terminal Region of the EGFR Is Critical to AR-Induced DNA Synthesis**—To understand better the role of the COOH-terminal region of the EGFR in mitogenic signaling, we assessed the ability of various concentrations of AR and EGF to stimulate DNA synthesis in serum-starved parental NR6, WT, and c’1000 cells (Fig. 1). As expected, neither AR nor EGF had any effect on DNA synthesis in NR6 parental cells (Fig. 1, top panel), whereas cells expressing WT EGFR acquired the ability to respond to both AR or EGF (middle panel). In cells WT the approximate concentration of growth factor necessary to achieve one-half of the maximal DNA synthesis response (EC50) was ~0.12 nM (Fig. 1, bottom panel) demonstrating that removal of the 186 amino acid residues from the COOH terminus of the receptor had no significant effect on the ability of EGF to stimulate DNA synthesis. However, in striking contrast to EGF, AR was impaired in its ability to stimulate DNA synthesis in c’1000 cells such that, relative to WT cells, the EC50 was shifted 67-fold to ~20 nM (bottom panel). Furthermore, no significant increase in DNA synthesis occurred in c’1000 cells in response to AR until

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2 A. Wells, unpublished observations.

3 L. Wong, T. B. Deb, S. A. Thompson, A. Wells, and G. R. Johnson, unpublished observations.
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Truncation of the EGFR Has No Significant Effect on AR Binding—The simplest explanation for the defect in the ability of AR to stimulate DNA synthesis effectively in c’1000 cells is that the truncated EGFR does not bind AR with the same affinity as the WT receptor. To test this hypothesis we studied the ability of AR and EGF to inhibit the binding of $^{125}$I-EGF to intact cells at 4 °C (Table I). In these experiments the concentration of unlabeled ligand that was necessary to inhibit the specific binding of radiolabeled EGF by 50% ($IC_{50}$) was determined in each of the two cell lines. This analysis demonstrated that truncation of the cytoplasmic domain of the EGFR had no significant effect on the binding of AR to the extracellular domain of the receptor (Table I). As expected, WT and c’1000 receptors bound EGF with comparable affinities consistent with previous observations (40). Thus, the impaired ability of AR to drive DNA synthesis in c’1000 is not due to a defect in the binding of AR to the truncated receptor.

COOH-terminal Truncation of the EGFR Impairs the Ability of AR to Elicit Transactivation of Elk-1—Activation of mitogen-activated protein kinase (MAPK, extracellular signal-regulated kinase) plays a critical role in mitogenic signaling by receptor tyrosine kinases such as the EGFR (47). MAPK-mediated phosphorylation of the transactivation domain of the ternary complex factor Elk-1 results in binding of Elk-1 to the serum response element in the promoters of growth factor-induced genes and the stimulation of transcription (48, 49). To assess the ability of the two ligands to transactivate Elk-1, cells were cotransfected with a plasmid that constitutively expresses a fusion protein consisting of the DNA binding domain of the yeast protein Gal4 and the transactivation domain of Elk-1, along with a reporter plasmid that expresses luciferase under the control of five tandem repeats of the Gal4-binding element.

Table I

| Cell line | AR $^{b}$ IC$_{50}$ $^{a}$ | Apparent $K_{i}$ $^{a}$ | EGF $^{b}$ IC$_{50}$ $^{a}$ | Apparent $K_{i}$ $^{a}$ |
|-----------|-----------------|-----------------|-----------------|-----------------|
| WT        | 11              | 8.8             | 2               | 1.6             |
| c’1000    | 6               | 5.3             | 0.65            | 0.57            |

$^{a}$IC$_{50}$ and apparent $K_{i}$ values are expressed in nanomolar units.
$^{b}$The IC$_{50}$ was defined as the concentration of unlabeled ligand that inhibited specific binding of 100 pM $^{125}$I-EGF by 50%.
$^{b}$Apparent $K_{i}$ values were estimated using the Cheng-Prusoff correction (69) and the $K_{i}$ values for the binding of $^{125}$I-EGF to WT and c’1000 receptors (40).

**Fig. 1.** Effect of AR and EGF on DNA synthesis in NR6 parent, WT, and c’1000 cells. NR6 parental cells or those expressing either human wild type (NR6 WT) or truncated EGFR corresponding to amino acid residues 1–1000 (NR6-c’1000) were grown to confluence in 96-well plates, serum-starved and stimulated with various concentrations of AR (open circles) or EGF (closed circles). After 6 h, the cells were treated with $^3$H-thymidine for an additional 16 h. DNA was harvested, and the incorporation of $^3$H-thymidine into newly synthesized DNA was quantitated as described previously (23) and expressed as cpm/well. Data points represent the means ± S.E. of triplicate experiments.

The concentration was escalated to 3.3 nM, whereas a significant response was observed in WT cells at the lowest AR dose tested (41 pM). In c’1000 cells the EC$_{50}$ for growth factor-stimulated DNA synthesis was 167-fold higher for AR than EGF. These results demonstrate that the COOH-terminal region of the WT EGFR is critical to the ability of AR to generate a strong mitogenic signal in NR6 cells.
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Fig. 2. Ligand-induced transcriptional activation of Elk-1 in NR6-WT and NR6-c’1000 cells. NR6-WT (upper panel) or c’1000 cells (lower panel) were transfected with 0.5 µg of a reporter plasmid that expresses luciferase under the control of 5 tandem repeats of a Gal4-binding element (pFRLuc), 50 ng of a plasmid that constitutively expresses a fusion protein consisting of the transactivation domain of Elk-1 and the DNA binding domain of Gal4 and 100 ng of cytomegalovirus promoter-driven ß-galactosidase (pcDNA 3.1/His/lacZ). The serum-starved cells were stimulated with various concentrations of AR (open circles) or EGF (closed circles) for 6 h, cells were lysed, and luciferase and ß-galactosidase activities were measured. Luciferase activity is normalized to ß-galactosidase activity and data points represent the means ± S.E. of duplicate experiments.

whether the defect in the ability of AR to stimulate DNA synthesis and Elk-1 transactivation in c’1000 cells could be explained by a defect in MAPK activation, we performed a time course experiment to monitor the enzymatic activation of MAPK. To reveal a potential defect in AR signaling in c’1000 cells, we used a concentration of ligand (1 nM) that was mitogenic for EGF but not for AR (Fig. 1, bottom panel). Cells were lysed at various time points, and lysates were analyzed by Western blotting using antibodies that specifically detect the phosphorylated activated form of MAPK (Fig. 3). As expected, both 1 nM AR and EGF strongly activated MAPK in cells expressing WT EGFR, and this stimulation was still evident 7 h after exposure to the growth factors (lanes 2–11 and 13–22). In c’1000 cells, however, a relatively strong but transient MAPK activation was elicited by AR (lanes 24–33), whereas EGF generated a robust and much more persistent activation of MAPK (lanes 35–44). This transient activation of MAPK by AR in c’1000 cells was also observed when MAPK activity was measured in immune complex kinase assays (data not shown).

Thus, unlike EGF, AR requires the presence of the COOH-terminal region of the WT EGFR for sustained enzymatic activation of MAPK.

AR- and EGF-stimulated Tyrosine Phosphorylation of Cellular Proteins in WT and c’1000 Cells—As seen in Fig. 4, both AR and EGF evoked a concentration-dependent tyrosine phosphorylation of an ~170-kDa protein as detected in whole cell lysates (Fig. 4A, lanes 1–9), and anti-EGFR immunoprecipitations performed on lysates derived from WT cells exposed to either 10 nM AR or EGF confirmed this protein to be the WT EGFR (Fig. 4A, lanes 10–15). Anti-phosphorytosine analysis of lysates from c’1000 cells revealed a concentration-dependent phosphorylation of ~150-, ~185-, and ~210-kDa proteins in response to EGF (Fig. 4B, lanes 6–9). AR induced tyrosine phosphorylation of the ~185-kDa protein, and a very low level of phosphorylation of the ~150- and ~210-kDa proteins was also observed (Fig. 4B, lanes 1–5). Anti-EGFR immunoprecipitations demonstrated EGF-stimulated tyrosine phosphorylation of c’1000, whereas no c’1000 tyrosine phosphorylation was detected in response to AR under these conditions (Fig. 4B, lanes 10–15). The c’1000 receptor contains one minor auto-phosphorylation site at Tyr-992 (36) which becomes tyrosine-phosphorylated in an EGF-dependent manner when expressed in NR6 cells (40). Co-migration of the immunoprecipitated c’1000 receptor (Fig. 4B, lanes 12–15) with the ~150-kDa phosphoprotein detected in lysates (lanes 8 and 9) suggests that the ~150-kDa lysate protein is the c’1000 receptor.

The Role of the COOH-terminal Region of the EGFR in Ligand-induced Coupling of GRB2 to Signaling Molecules—A major pathway implicated in mitogenic signaling by the EGFR involves the recruitment of the adaptor protein GRB2 and the guanine nucleotide exchange factor Son of sevenless to the plasma membrane where activated Ras initiates a kinase cascade which proceeds downstream to activate MAPK (50). Due to the fact that the adaptor protein GRB2 plays such a crucial role in the activation of MAPK and mitogenic signaling by the EGFR, we performed a time course experiment to monitor the association of GRB2 with a number of signaling molecules. As before, to reveal potential defects in AR signaling in c’1000 cells, we used a concentration of ligand (1 nM) that was mitogenic for EGF but not for AR. Cells were lysed at various time points after treatment and probed with a GRB2-GST fusion protein (Fig. 5A).

Analysis by Western blotting of proteins that bound to GRB2-GST demonstrated that AR or EGF induced binding of GRB2 to the EGFR only in cells expressing the full-length receptor (lanes 1–9), whereas no association between GRB2 and c’1000 receptor was detected (lanes 10–18). Furthermore, no coupling of the EGFR-related tyrosine kinase ErbB2 to GRB2 was detected in WT cells in response to either ligand (lanes 2–9), and coupling was also not observed when the dose of AR and EGF was increased to 10 nM (data not shown). AR was not able to induce an interaction between ErbB2 and GRB2 in c’1000 (lanes 11–14), whereas EGF stimulated the association of ErbB2 and GRB2 in c’1000 cells that persisted for at least 30 min in the presence of ligand (lanes 15–18). Therefore, the EGF-activated c’1000 receptor drives the association of ErbB2 and GRB2 into a protein complex.

Previous work has demonstrated that the catalytic activity and both SH2 domains of the protein-tyrosine phosphatase SHP-2 are essential for MAPK activation by the entire ErbB family of receptors (51), and EGF stimulates the recruitment of SHP-2 into a protein complex with GRB2 via the COOH-terminal Src homology 3 (SH3) domain of GRB2 (52). The presence of the COOH-terminal region of the EGFR was found to be essential for AR-induced coupling of SHP-2 to GRB2, since AR was only able to
elicit a significant association in WT cells (lanes 2–5) but not in c'1000 cells (lanes 11–14). However, EGF stimulated the association between GRB2 and SHP-2 in both cell lines (lanes 6–9 and 15–18). We also evaluated the interaction between GRB2 and SHC that occurs via an interaction between the SH2 domain of GRB2 and tyrosine-phosphorylated SHC. Interestingly, removal of the COOH-terminal region of the EGFR had little effect on this interaction in response to AR since levels of SHC (p46 and p52) which bound to GRB2-GST were comparable in both cell lines (lanes 2–5 and 11–14). For EGF, enhanced association of GRB2 and SHC was observed in c'1000 relative to WT cells (lanes 6–9), and in particular, coupling of the p66 SHC isoform was significantly elevated (lanes 15–18). Taken together, the data presented in Fig. 5A revealed that truncation of the cytoplasmic tail of the EGFR resulted in defective AR-induced GRB2 adaptor function.

EGF Stimulates Association of ErbB2 with SHC in c’1000 Cells—To provide further insight into the potential role of ErbB2 in signaling by WT and c’1000 receptors, we analyzed ligand-induced association of ErbB2 and SHC. Both AR and EGF were capable of stimulating ErbB2 tyrosine phosphorylation in WT and c’1000 cells, but no striking differences were observed (data not shown). SHC interacts with and is an excellent substrate for ErbB2 and appears to play an important role in mitogenic signaling through this receptor tyrosine kinase (53). We therefore assessed the ability of SHC to associate with ErbB2 in a ligand-dependent manner in WT and c’1000 cells. In these experiments, cells were exposed to the growth factors, SHC was immunoprecipitated, and the immunoprecipitates were probed for the presence of SHC, ErbB2, and phospho-tyrosine-containing proteins by Western blotting (Fig. 5B). No induced coupling of ErbB2 to SHC could be detected in WT cells (lanes 2–4). Western blotting analysis for phospho-tyrosine-containing proteins did demonstrate association of SHC with the highly tyrosine-phosphorylated ~170-kDa WT EGFR in response to AR and EGF (Fig. 5B, lanes 2–4). In c’1000 cells, it was possible to demonstrate EGF- but not AR-induced association between SHC and ErbB2, albeit at barely detectable levels (lanes 7 and 8). Anti-phosphotyrosine analysis confirmed the presence of a protein in the SHC immunoprecipitates that co-migrated exactly with a ~185-kDa ErbB2 (lanes 7 and 8). Furthermore, EGF elicited the complex formation between SHC and additional phosphotyrosine-containing proteins of ~150 and ~210 kDa (lanes 7 and 8). Reprobing of this blot with antibodies directed against the EGFR extracellular domain was not able to confirm that the ~150-kDa protein was the c’1000 receptor (data not shown). Thus, in the absence of the COOH-terminal region of the WT EGFR, EGF stimulates the generation of a complex that contains both ErbB2 and SHC.
terminal tail that contains the tyrosine phosphorylation sites. Cells were transfected with the ErbB2 cDNAs, and as seen in Fig. 6A transient expression of WT ErbB2 in c’1000 cells resulted in a strong activation of Elk-1 that occurred in the absence of any exogenous growth factor. In contrast, expression of WT ErbB2 in the NR6 parent or WT cells only modestly increased Elk-1 transactivation demonstrating that the strong Elk-1 activation observed in c’1000 cells by WT ErbB2 was due to the presence of the c’1000 receptor in these cells. Expression of ErbB2-(1–813) had no significant effect on ligand-independent Elk-1 transactivation in the three cell lines (Fig. 6A) indicating that the ErbB2 kinase activity and/or COOH-terminal region was required for the constitutive Elk-1 activity by the c’1000 EGFR. To confirm consistent expression of the ErbB2 structures and overexpression of the ErbB2s relative to endogenous murine ErbB2, pooled lysates derived from the experiments shown in Fig. 6, A and C, were analyzed for ErbB2 expression by Western blotting with two different monoclonal antibodies (Fig. 6B). The lower panel of Fig. 6B was probed with an antibody that cross-reacts with both human and mouse ErbB2 and is directed against the COOH terminus of rat ErbB2 (c-neu) amino acid residues 1242–1255). This analysis demonstrated that the transfected WT ErbB2 was expressed at comparable levels in all three cell lines and confirmed that WT ErbB2 was overexpressed relative to endogenous murine ErbB2. The upper panel of Fig. 6B was probed with an antibody that is specific to the extracellular domain of human ErbB2 and does not detect mouse ErbB2. These data demonstrated that both WT ErbB2 (lanes 2, 5, and 8) and ErbB2-(1–813) (lanes 3, 6, and 9) were expressed at comparable levels in each of the cell lines confirming that the ligand-independent transactivation of Elk-1 by WT ErbB2, but not by ErbB2-(1–813), in c’1000 cells was specific and not due to differential levels of expression of the two ErbB2 proteins. Taken together, these results reveal that when ErbB2 is overexpressed in the presence of the c’1000 receptor, downstream signaling can occur in the absence of ligand and suggests that c’1000, relative to WT EGFR, has an enhanced ability to signal through ErbB2.

Overexpression of ErbB2-(1–813) Inhibits Elk-1 Transactivation by EGF but Not by Activated Ras—Since the data suggested that the EGF-activated c’1000 EGFR was signaling through ErbB2, we tested the ability of the ErbB2-(1–813) to inhibit ligand-induced Elk-1 transactivation and to thus act as a dominant-negative structure. Overexpression of ErbB2-(1–813) or WT ErbB2 in WT EGFR cells had no significant effect on Elk-1 activation which occurred in response to 10 nM AR or EGF (Fig. 6C). Conversely, expression of ErbB2-(1–813) in c’1000 cells inhibited EGF-stimulated Elk-1 transactivation by ~54% (Fig. 6C). In addition, ErbB2-(1–813) also modestly inhibited (~29%) the weaker Elk-1 activation that was observed in response to 10 nM AR (Fig. 6C). To confirm the specificity of the ErbB2-(1–813) dominant-negative effect and to demonstrate that ErbB2-(1–813) functioned upstream of Ras, we tested the ability of it to influence activated Ras-mediated Elk-1 transactivation (Fig. 6C, bottom). In these experiments the cells were also transfected with either 50 ng of a constitu-
tively activated form of Ras (Q61L) or empty vector. As seen in Fig. 6C neither WT ErbB2 nor residues 1–813 had any significant effect on Elk-1 activation by Ras Q61L in either NR6-WT or c'1000 cells. These results confirm that the ErbB2-(1–813) dominant-negative effect in c'1000 cells is specific and is consistent with an interference of EGF-activated c'1000 signaling between the c'1000 receptor and Ras. In conclusion, these findings demonstrate that endogenous ErbB2 plays a critical role in ligand-induced signaling by the c'1000 but not by the WT EGFR in NR6 cells.

**DISCUSSION**

In the present study we have investigated the mitogenic signaling by AR and EGF in EGFR-devoid NR6 fibroblasts that ectopically express either WT EGFR or a COOH-terminal truncated EGFR (c'1000) that lacks the three major sites of autophosphorylation. Here we demonstrate that the last 186 amino acid residues in the cytoplasmic tail of the EGFR are critical to and play an important role in AR-mediated mitogenic signaling. It should be noted that AR can elicit some signaling when very high, non-physiological concentrations of AR were used (i.e., 10 nM). However, when compared with the WT EGFR, the ability of AR to stimulate DNA synthesis, generate a sustained MAPK activation, and to induce Elk-1 transactivation through the c'1000 receptor was weak and defective. Furthermore, the data imply that defective GRB2 adaptor function may form the molecular basis for the weak AR-induced signaling observed in
The effect of transient overexpression of ErbB2 or truncated ErbB2 on ligand-independent and -dependent Elk-1 trans-activation in NR6-WT and c'1000 cells. Parental mouse NR6 (parent), NR6-WT, or NR6-c'1000 cells were transfected with 0.5 μg of pFR-Luc, 50 ng of pFA2-Elk-1, 100 ng of pcDNA 3.1/His/ lacZ, and 1.35 μg of pcDNA 3.1 (empty vector) or pcDNA 3.1 encoding either full-length human ErbB2 (WT) or ErbB2-(1–813). The cells were allowed to recover overnight in serum-containing medium and were serum-starved. A, cells were lysed without growth factor treatment, and luciferase and β-galactosidase activities were measured. B, pooled lysates derived from identically transfected dishes were analyzed for ErbB2 expression by Western blotting (WB) using either a monoclonal antibody that is specific to the extracellular domain of human ErbB2 or a monoclonal antibody that detects the COOH terminus (amino acid residues 1242–1255) of both mouse and human ErbB2. C, serum-starved cells were stimulated for 6 h with 10 nM AR or EGF, lysed, and luciferase and β-galactosidase activities were measured. In the bottom bar graph of C, cells were also transfected with 50 ng of a cDNA construct encoding a constitutively active form of Ras (Q61L) (+) or empty vector (−), and cells were lysed without growth factor treatment. Luciferase activity is normalized to β-galactosidase activity, and data points represent the means ± S.E. of duplicate experiments.
cells expressing the truncated EGFR. Conversely, removal of the COOH-terminal region of the EGFR had no significant effect on mitogenic signaling by EGFR consistent with the previous findings of others (39–44). The goal of this study was to attempt to understand the molecular basis for this differential signaling by AR and EGFR. The fact that, unlike EGFR, AR requires the presence of heparan sulfate proteoglycan for receptor binding and full bioactivity (16) cannot explain the defective AR signaling in c’1000, because no significant difference in ligand binding to receptors on WT and c’1000 cells was observed. EGF-stimulated tyrosine phosphorylation of Tyr-992 in c’1000 cells has been demonstrated previously (40). In contrast to EGFR, we observed no significant AR-induced tyrosine phosphorylation of the c’1000 receptor. Regardless, tyrosine phosphorylation of Tyr-992 has been shown not to be required for EGFR-mediated mitogenesis but is essential for phospholipase C-γ activation, motility (40, 54), and attenuation of EGFR mitogenic signaling by a phospholipase C-γ/protein kinase C feedback mechanism (55).

The most striking signal that was generated by EGF in c’1000, but not in WT cells, was the association of GRB2 and SHC with the EGFR-related tyrosine kinase, ErbB2. Unfortunately, attempts to detect chemically cross-linked ligand-induced c’1000 and ErbB2 heterodimers was not successful (data not shown), most probably due to the relatively low levels of ErbB2 in NR6 cells and/or the inefficiency of the cross-linking process. It has long been known that EGFR ligands can induce tyrosine phosphorylation of ErbB2 (11, 56–59) and that EGFR and ErbB2 can cooperate in signaling and malignant transformation of cells via heterodimerization (60, 61). A possible role for ErbB2, in EGF-induced MAPK activation and mitogenic signaling by EGFR mutants, has been proposed previously (43, 62). However, the role of ErbB2 in WT EGFR signaling is somewhat controversial. The present data indicate that ErbB2 is critical for signaling from EGFRs lacking the COOH-terminal tail but suggest that ErbB2 is not required for full-length WT EGFR signaling. This finding is consistent with previous studies demonstrating that ectopic expression of WT EGFR in hematopoietic 32D or BAF3 cells, which do not express ErbB2, results in the acquisition of EGF-mediated mitogenic signaling by these cells (63, 64).

Since SHC and GRB2 appear to be involved in the activation of Ras by ErbB2 (53, 65, 66), we hypothesized that the EGF-stimulated interactions of GRB2 and SHC with ErbB2 in c’1000 cells may supplant the requirement for GRB2-SHC interactions with the COOH-terminal region of the EGFR in WT cells. To demonstrate a causal relationship between ErbB2 and EGF mitogenic signaling in c’1000 cells, we transiently expressed either WT ErbB2 or a truncated ErbB2-(1–813) in the WT and c’1000 cells and studied ligand-dependent and -independent Elk-1 activation. Greene and co-workers (67) have used an ErbB2 molecule truncated at residue 691 to suppress cellular transformation mediated by oncogenic ErbB2 (neu), as well as the malignant phenotype of human glioblastoma cells (68). In our study overexpression of full-length ErbB2 resulted in ligand-independent signaling and transactivation of Elk-1 in c’1000 but not in parental or WT NR6 cells. This indicated that removal of the COOH-terminal region of the EGFR results in a receptor (c’1000) that has a propensity to interact with ErbB2 and supports the notion that the cytoplasmic tail of the EGFR functions to block the inappropriate interaction of the EGFR with ErbB2 (i.e., in the absence of ligand).

The most convincing result to demonstrate that the potent EGF stimulated signaling by c’1000 receptor was occurring through ErbB2 was our finding that expression of ErbB2-(1–813), which lacks the kinase domain and COOH-terminal phosphorylation sites, functions as a dominant-negative and specifically inhibited EGF signaling in c’1000 but not WT cells. Western blotting analysis of lysates demonstrated that NR6 cells express relatively low to moderate levels of ErbB2 when compared with recombinant cell lines engineered to express ErbB2 or human breast or ovarian carcinoma cells such as SK-Br-3 or SK-OV-3.3 WT cells express ~100,000 EGFRs per cell (40). Thus, the fact that ErbB2-(1–813) does not inhibit either AR or EGF signaling in WT cells is most likely due to the fact that the truncated ErbB2 molecule is not expressed at a high enough level to compete effectively with ligand-induced homodimerization of full-length EGFRs. Conversely, ErbB2-(1–813) can compete with the relatively low to moderate levels of endogenous ErbB2 in NR6 cells, and the EGF-activated c’1000 receptor needs to interact with endogenous ErbB2 for strong signaling. We cannot rule out the possibility that endogenous ErbB2 is involved in AR and EGF signaling in WT cells, but ErbB2 clearly plays an important role in EGF mitogenic signaling by the truncated EGFR, c’1000.

The differential requirement for the COOH-terminal region of the EGFR in AR and EGF signaling implies a certain degree of ligand-specific diversity in the signal transduction mechanisms of the EGFR. Clearly, the COOH-terminal region of the EGFR is essential for efficient mitogenic signaling by AR, and removal of this region results in a number of signaling defects that have a molecular basis (i.e. GRB2 adaptor function). Furthermore, our findings suggest that receptor homo-oligomerization is a fundamental requirement for AR function. The fact that AR requires HSPGs to activate the EGFR (16) suggests that HSPGs may be involved in preferentially guiding the AR-bound EGFR into homo-oligomers relative to hetero-oligomers. On the other hand, EGF mitogenic signaling does not require the EGFR cytoplasmic tail, and in the absence of this region, the EGF-activated truncated EGFR finds an additional mechanism to signal, and this signaling necessitates ErbB2 function. This may reflect a greater degree of redundancy in the ability of EGF to activate specific mitogenic signaling pathways and linked protein-protein interactions. Our results also suggest that EGF, when compared with AR, has a greater tendency to elicit hetero-oligomerization of the EGFR with ErbB2. The findings that we have presented here support the concept that studies involving EGFR mutants and different EGFR ligands may provide important insights into the mechanisms that the EGFR utilizes to evoke downstream signaling events.

REFERENCES

1. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D., and Seeburg, P. H. (1984) Nature 309, 418–425
2. Coussens, L., Yang-Feng, T. L., Liao, Y.-C., Chen, E., Gray, A., McGrath, J., Seeburg, P. H., Libermann, T. A., Schlessinger, J., Francke, U., Levinson, A., and Ullrich, A. (1985) Science 230, 1132–1139
3. Krauss, M. H., Ising, W., Malsch, T., Popescu, N., and Aaronson, S. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9193–9197
4. Plowman, G. D., Coloucouzou, J.-M., Whitney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neuhauer, M. G., and Shoyab, M. (1990) Proc. Natl. Acad. Sci. U. S. A. 90, 1746–1750
5. Lemmon, M. A., and Schlessinger, J. (1994) Trends Biochem. Sci. 19, 459–463
6. Savage, C. R., Jr., Inagami, T., and Cohen, S. (1972) J. Biol. Chem. 247, 7612–7621
7. Carpenter, G., and Cohen, S. (1990) J. Biol. Chem. 265, 7709–7712
8. Massague, J. (1983) J. Biol. Chem. 258, 13614–13620
9. Marquart, H. M., Hunkapiller, W., Hood, L. E., and Todaro, G. J. (1984) Science 223, 1079–1082
10. Shoyab, M., Plowman, G. D., McDonald, V. L., Bradley, J. G., and Todaro, G. J. (1989) Science 243, 1074–1076
11. Johnson, G. R., Kannan, B., Shoyab, M., and Stromberg, K. (1993) J. Biol. Chem. 268, 2924–2931
12. Thompson, S. A., Harris, A., Hoang, D., Ferrer, M., and Johnson, G. R. (1996) J. Biol. Chem. 271, 17927–17931
13. Higashiyama, S., Abraham, J. A., Miller, J., Fiddles, J. C., and Klagsbrun, M. (1992) Science 251, 936–939
14. Shing, Y., Christofori, G., Hanahan, D., Ono, Y., Sasada, R., Igarashi, K., and
