ABSTRACT

The epidermal growth factor-like receptor tyrosine kinase (ErbB) family is frequently overexpressed in a variety of human carcinomas, including breast cancer. To assist in characterizing the role of ErbB-4 in breast cancer, we generated three specific hammerhead ribozymes targeted to the ErbB-4 mRNA. These ribozymes, Rz6, Rz21, and Rz29, efficiently catalyzed the specific cleavage of ErbB-4 message in a cell-free system. We demonstrated that the neuregulin-induced mitogenic effect was abolished in ribosome Rz29- and Rz6-transfected 32D/ErbB-4 cells. Inhibition of mitogenesis was characterized by ribozyme-mediated down-regulation of ErbB-4 expression. In addition, we provide the first evidence that different threshold levels of ErbB-4 expression and activation correlate with different responses to neuregulin stimulation. High levels of ErbB-4 expression, phosphorylation, and homodimerization are necessary for neuregulin-stimulated, interleukin 3-independent cell proliferation in the 32D/E4 cells. In the case of Rz29-transfected 32D/E4 cells, low levels of ErbB-4 expression allowed neuregulin-induced phosphorylation but were insufficient to couple the activated receptor to cellular signaling. Furthermore, expression of the functional ErbB-4 ribozyme in T47D human breast carcinoma cells led to a down-regulation of endogenous ErbB-4 expression and a reduction of anchorage-independent colony formation. These studies support the use of ErbB-4 ribozymes to define the role of ErbB-4 receptors in human cancers.

INTRODUCTION

The EGFR/ErbB family is a group of tyrosine kinases that is frequently overexpressed in a variety of carcinomas (1-3). This class I subfamily of receptors is composed of four members: EGFR (4); HER2/ErbB-2/neu (5); HER3/ErbB-3 (6, 7); and HER4/ErbB-4 (8). Data from numerous laboratories suggest that the EGFR family members may play a complex role in signaling (9-11). Most human breast cancer cells express more than one of the EGFR family receptors, and different combinations of receptors can heterodimerize or homodimerize. These receptor interactions lead to the activation of multiple signaling pathways and contribute to the pathogenicity and tumorigenicity of breast cancer (12). A number of growth factors, classified as EGF-like ligands, have been identified that bind and stimulate the kinase activity of EGFR family receptors. EGF, transforming growth factor α, amphiregulin, heparin-binding EGF, and betacellulin have been described as specific for EGFR (13-17). Several differentially spliced variants, named NRG1-α/HRG, or neu differentiation factor (18, 19), acetylcholine-receptor inducing activity (20), glial growth factor (21), and gp30 (22) were initially identified as candidate neu ligands because of their ability to induce neu tyrosine phosphorylation. However, recent results demonstrate that ErbB-3 and ErbB-4 are primary receptors for neuregulin (23, 24). Activation of ErbB-2 by NRG1-α is thought to occur through transphosphorylation resulting from heterodimerization with either ErbB-3 or ErbB-4 (25-27). Most recently, betacellulin has been shown to activate the ErbB-4 receptor in a Ba/F3 system (28); heparin binding-EGF can bind and activate ErbB-4 as well (29).

Amplification and/or overexpression of EGFR and ErbB-2 are clearly important factors in neoplastic transformation of breast epithelium (30). Elevated ErbB-4 levels have been found in certain breast cancer cell lines (8), but little is known about the expression or the clinical significance of ErbB-4 receptors in the diagnosis and prognosis of human breast cancer. It is therefore imperative that the role of ErbB-4 and its biological significance in breast cancer be defined. To achieve this goal, we used ribozyme technology to disrupt ErbB-4 expression in human breast cancer cells.

Specific gene modulation using oligonucleotides, including triplex DNA, antisense DNA/RNA, and ribozymes, have been used as strategies for suppressing activated oncogenes (31-33). In the present study, we generated three specific hammerhead ribozymes targeted to specific sites within ErbB-4 mRNA. These ErbB-4 ribozymes (Rz6, Rz21, and Rz29) effectively catalyzed the precise cleavage of ErbB-4 mRNA under physiological conditions in a cell-free system. One of these ribozymes, Rz29, down-regulated ErbB-4 receptor expression by as much as 65%, with a corresponding 10-fold decrease in ErbB-4 tyrosine phosphorylation in a 32D cell model system. Furthermore, expression of this functional ErbB-4 ribozyme in T47D human breast carcinoma cells led to a down-regulation of endogenous ErbB-4 expression and a reduction of anchorage-independent colony formation.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The 32D murine hematopoietic cell line (34) and its derivatives were grown in RPMI (Cellgro) supplemented with 12% FCS (Biowhittaker) and IL-3 supplied as 6% conditioned medium from the WEHI-3B murine myelomonocytic leukemia cell line.

Generation of ErbB-4 Ribozymes. We used the GCG Package Database program to select the ribozyme sequence: (a) we used this program to predict the optimal and suboptimal secondary structure of ErbB-4 mRNA using the most recent energy minimization method by Zuker and Stiegler (36); (b) we selected ribozyme target sites in the open loop regions with the GUX cleavage site; (c) we then used the same program to predict the secondary structure of the selected regions to see whether these sequences are able to fold into a typical hammerhead ribozyme three-loop structure; and (d) we tested the selected ribozyme sequences for specificity against other known human genes in the GenBank database.

Plasmid Construction. Two synthetic single-stranded ribozyme oligonucleotides were subcloned into the mammalian vector pCR3. The sequence and orientation of the inserts were confirmed by dyeoxynucleotide sequencing of the construct using the Sequenase kit, version 2.0 (U.S. Biochemical Corp., Cleveland, OH). ErbB-4 ribozyme sequences were: Rz6, 5'-AUU UGC GCU CAC CCA CUG AUG UCG UGAA GGA CGA CAA CCA AAC UCC C-3'; Rz21, 5'-UUU UGC UUG CCC AUC UGA UGA GUC G CU GAG GAC GAA ACA ACC UCA CC-3'; and Rz29, 5'-AUU UCC ACU AAC ACG CUG AUG AGU CCG UGA GGA CGA AAC UCC GCU GU C-3'.
Ribosome-mediated mRNA Cleavage in Vitro. The substrate ErbB-4 cDNA fragment was derived by reverse transcription-PCR with RNA from MDA-MB-453 cells, which express relatively high levels of ErbB-4. The PCR primers for subcloning of ErbB-4 cDNA were: 5' primer sequence, 5'-AAT TGT CAG CAC GGG ATC TGA GAC-3'; and 3' primer sequence, 5'-GTT TCC TTA AAC AAG ACC AGA TGT-3'. The reverse transcription-PCR products were then cloned into the PCR3 vector. Clones were sequenced to verify that they contained the ErbB-4 cDNA fragment. We then performed in vitro run-off transcripts from an ErbB-4 cDNA construct to generate the ErbB-4 ribozyme substrate. Likewise, ribozymes were chemically synthesized as DNA oligonucleotide and subsequently synthesized in vitro by using the T7 RNA polymerase. Cleavage reactions were performed in 50 mM Tris-Cl (pH 8.0) and 20 mM MgCl₂. Substrate and ribozyme reactions were then mixed and incubated at 37°C for 30 min. Reaction products were analyzed on 6% polyacrylamide gel, and products were detected by autoradiography.

Transfection by Electroporation. 32D derivative cells (1 × 10⁷) were used for each transfection. Ten μg of plasmid DNA were added to cells resuspended in 300 μl of PBS. Cells were electroporated at 250 V, using a Bio-Rad electroporation system, plated onto 100 mm dishes, and incubated for 24 h. The cells were then selected in growth medium containing 750 μg/ml geneticin (G418-sulfate; Life Technologies, Inc.).

Northern Blot Analysis. Total RNA from cell cultures was isolated using RNasol B (Tel-Test, Inc., Friendswood, TX). Twenty μg of total RNA from each cell line were used to hybridize with an ErbB-4 cDNA probe and autoradiographed for 48 h.

Autophosphorylation of erbB Family Receptors. A total of 2 × 10⁶ 32D derivative cells were washed in PBS and resuspended in 50 ml of RPMI supplemented with IL-3 and incubated for 4 h at 37°C. After incubation, cells were washed in PBS and resuspended in 1 ml of PBS with 10 μg/ml actinomycin D. Remaining steps were performed on ice. Recombinant HRG-B3 isoform (EGF-like domain) was added at a final concentration of 150 ng/ml. After a 10-min incubation, cells were lysed in a HEPES-lysis buffer, and the cell debris was pelleted by centrifugation (28).

The lysates were then immunoprecipitated with either anti-EGFR (Ab-1; Oncogene Science, Uniondale, NY), anti-ErbB-2 (Ab-2; Oncogene Science), anti-ErbB-3 (C17; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-ErbB-4 (C18; Santa Cruz Biotechnology) in combination with protein A-agarose (Pharmacia, Piscataway, NJ) overnight at 4°C with gentle agitation. For details, see Rieke et al. (28).

FACS (FACStar) Analysis. Cells (1 × 10⁶) were harvested and then stained for 1 h with either anti-EGFR (Ab-1; Oncogene Science), anti-ErbB-2 (Ab-2; NeoMarker, Fremont, CA), anti-ErbB-3 (Ab-4; NeoMarker), and anti-ErbB-4 monoclonal antibody (Ab-1; NeoMarker); then a secondary FITC-antimouse antibody was used, and the ErbB-4 level in each cell was quantitatively measured by flow cytometry.

Anchorage-independent Growth Assay. A bottom layer of 0.1 ml of IMEM containing 0.6% agar and 10% FCS was prepared in 35-mm tissue culture dishes. After the bottom layer solidified, cells (10,000 per dish) were added in a 0.8-ml top layer, 0.4% Bacto Agar and 5% FCS. All samples were prepared in triplicate. Cells were incubated for 12 days at 37°C. Colonies larger than 60 μm were counted in a cell colony counter (Omnimas 3600; Imaging Products International, Inc., Charley, VA).

Mitogenic Assay. 32D transfected cells were plated at a density of 1 × 10⁵ cells with or without IL-3 supplement or supplemented with 100 ng/ml of NGF1-α in the absence of IL-3. Two days after plating, the cells were labeled with [³H]thymidine for 2 h. [³H]Thymidine incorporation was then analyzed by β-scintillation counter.

In Vitro Kinase Assay. 32D/E4, 32D/E4 + V, and 32D/E4 + Rz29 cells were serum starved for 2 h before treatment with or without 100 μg/ml of NGF1-α. Cells then lysed in lysis buffer. Four hundred μg of total protein of each cell line was used to immunoprecipitate with anti-ErbB-3 antibodies (C18; Santa Cruz Biotechnology) in combination with protein A-agarose (Pharmacia, Piscataway, NJ). Reactions were carried as described previously (35). Briefly, 50 μl of a solution containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM MnCl₂, 100 μM of [γ⁻³²P]ATP, and 1 μg of aprotinin were added to the washed beads for 25 min at room temperature. Reactions were terminated by spinning down the Sepharose beads in a microcentrifuge, discarding the supernatant, and resuspending the beads in 50 μl of SDS gel loading buffer. Eluted proteins were analyzed by SDS-PAGE and autoradiography.

RESULTS

Generation and Demonstration of ErbB-4 Ribozyme Efficacy and Specificity in a Cell-free System

To investigate the biological significance of ErbB-4 in human breast cancer cells, we used molecular targeting of the ErbB-4 mRNA by ribozymes. Three ribozymes (Rz6, Rz21, and Rz29) targeted to specific sites within the ErbB-4 mRNA open reading frame were generated. These ribozymes were modeled on the hammerhead structure described previously (36, 37), derived and minimized to the catalytic center portion of 22 nucleotides. The targeted cleavage sites selected for the design of the ribozymes were 60 (Rz6), 210 (Rz21), and 290 (Rz29) nucleotides downstream of the translation initiation site of the ErbB-4 mRNA (Fig. 1). The catalytic activity of these ribozymes was first evaluated in an extracellular system. All three ErbB-4 ribozymes cleaved ErbB-4 mRNA precisely and efficiently under physiological conditions in this cell-free system (Fig. 2A, Lanes 2–5). Cleavage was specific because the actual sizes of the cleaved fragments correspond to the expected sizes if cleavage were to occur immediately 3' to the GUN sequence. As a control for specificity, catalytically inactive mutant ribozymes were engineered. Point mutation of G to A in the catalytic domain of either Rz29 or Rz6 (Fig. 2A, Lanes 6 and 7) resulted in loss of catalytic activity as predicted by mutational studies of McCall et al. (37) reported previously. The specificity of these three ErbB-4 ribozymes was evaluated on a nonspecific mRNA substrate. As expected, no cleavage was observed, following incubation of these ribozymes with ErbB-3 mRNA (Fig. 2B). These results indicate that all three of the GUN sequences chosen in the ErbB-4 mRNA are accessible to ribozyme-mediated cleavage in an extracellular system.

An Intracellular Model System for Evaluating the Specificity and Efficacy of ErbB-4 Ribozymes. We next investigated the catalytic activity of these ribozymes in a model cellular system. Although the ribozyme sensitivity in an extracellular system can be correlated with the predicted secondary structure of the target RNA, the intracellular susceptibility of the target RNAs to ribozymes does not necessarily correlate with their predicted secondary structure. In addition, the complexity of heterodimerization and transphosphorylation between the ErbB family members in breast cancer cells makes it difficult to determine the specificity of ErbB-4 ribozymes. Furthermore, the goal of these ribozymes is to interrupt gene expression. If ErbB-4 is critical for cell proliferation, its down-regulation may be lethal. Thus, an ideal system for screening the intracellular enzymatic activity of these ribozymes requires the following criteria: (a) expression of high levels of ErbB-4 receptor; (b) no expression of other EGF family receptors; (c) nonlethality of ErbB-4 ribozyme introduction; and (d) easy detection of ribozyme activity by bioassay. We therefore used the 32D cell system to examine the intracellular efficacy and specificity of the ErbB-4 ribozymes. 32D cells are a murine hematopoietic IL-3-dependent cell line that does not express detectable levels of endogenous EGF family receptors. Studies have shown that IL-3...
Fig. 2. A. catalytic activity of ErbB-4 ribozyme in an extracellular system. Lane 1, molecular weight markers. Lane 2, 32P-labeled ErbB-4 transcript with an expected size of 622 nucleotides. Lanes 3–5, cleavage products of the three ErbB-4 ribozymes (Rz6: 285, 337 nucleotides; Rz21: 232, 390 nucleotides). In Lanes 6 and 7, mutant ribozymes do not cleave ErbB-4 transcript. B: Lane 1, molecular weight marker. Lane 2, 32P-labeled ErbB-3 transcript with an expected size of 698 nucleotides. In Lanes 2–4, ErbB-3 transcript is not cleaved by ErbB-4 ribozymes.

Demonstration of ErbB-4 Ribozyme Catalytic Activity in 32D Cells

ErbB-4 Ribozymes Abolish NRG1-α-induced IL-3 Independence. All three ErbB-4 ribozymes were cloned into a mammalian expression vector downstream of the cytomegalovirus early promoter. We then transfected the ErbB-4Rz into 32D/E4 cells. We hypothesized that the functional ribozymes would down-regulate ErbB-4 expression and thereby reduce or abolish the NRG1-α-induced, IL-3-independent survival or proliferation. ErbB-4Rz-transfected cells were tested for growth in the presence and absence of NRG1-α. Cell lines expressing one of the ErbB-4 ribozymes (Rz29) failed to respond to NRG1-α and proliferated in an IL-3-dependent manner. In contrast, parental 32D/E4 and vector alone-transfected cells responded to NRG1-α and proliferated in the absence of IL-3. Rz6 partially inhibited the NRG1-α effect. In contrast, Rz21 had no effect on responsiveness to NRG1-α stimulation. Table 1 summarizes the ribozyme effects in these ErbB-4 cells. We next evaluated the specificity of the

Biological Function of EGF Family Receptors in 32D Cells. 32D cell transfectants express the EGF receptor family members individually and in pairwise combinations (34). The resultant stably transfected cells were designated as 32D/E1, 32D/E2, 32D/E3, 32D/E2 + E3, and 32D/E4, where E1, E2, E3, and E4 refer to EGFR, ErbB-2, ErbB-3, and ErbB-4 receptors, respectively. The high levels of receptor expression were confirmed by Western blotting or immunoprecipitation followed by Western blotting (data not shown). No detectable levels of endogenous EGF family receptor expression were found in parental 32D cells. In the absence of cognate ligands, all of the 32D transfected cells remained dependent on IL-3 for survival (39). 32D transfected cells were tested for induction of IL-3-independent survival or proliferation. Consistent with previous studies (34, 39), untransfected parental cells did not proliferate or survive after NRG1-α stimulation. Cells transfected with ErbB-4 or coexpressing ErbB-2 and ErbB-3 bypassed the IL-3-dependent pathway in response to NRG1-α stimulation, but cells transfected with ErbB-2 or ErbB-3 alone did not survive and proliferated in an IL-3-dependent manner (Fig. 3). Regulation of tyrosine phosphorylation of each receptor by NRG1-α was evaluated by immunoprecipitating the corresponding receptors and immunoblotting with antiphosphotyrosine. Fig. 4 demonstrates that no autophosphorylation was observed in the parental cells (32D) in the presence of NRG1-α. In both EGFR- and ErbB-4expressing cells, the receptors were constitutively phosphorylated; however, phosphorylation could be further induced after exposure to its cognate ligands. In 32D/E2 cells, a marginal phosphorylation of ErbB-2 was observed in the absence of NRG1-α, but receptor phosphorylation was not elevated in the presence of NRG1-α (Fig. 4). No phosphorylation was observed in the presence or absence of NRG1-α in 32D/E3 cells. In 32D/E2 + E3 cells, a high basal level of phosphorylated ErbB-3 was observed, and increased phosphorylation was observed after NRG1-α stimulation (Fig. 4). Thus, the 32D cells provide an ideal system to study the specificity and efficacy of the ribozymes targeting the ErbB family receptors.

Fig. 3. Growth assay. 32D cells were plated at a density of 1 × 10⁵ cells/ml in IL-3-free medium, medium supplemented with IL-3, or in medium lacking IL-3 but supplemented with 100 ng/ml of human recombinant NRG1-α. Viable cells were counted on day 3 after seeding. NRG can induce IL-3-independent growth in 32D/E4 and 32D/E2 + E3 cells. All samples were prepared in triplicate. This assay was repeated more than three times. The SD was within 10%. ■ IL-3; ■, no IL-3; □, NRG.
ErbB-4 ribozymes by expressing all three ErbB-4 ribozymes in 32D/E2 + E3 cells. No effect on the NRG1-α-induced IL-3-independent survival and proliferation was observed. We then evaluated the efficacy of the ribozyme by using an ErbB-2 ribozyme, which has been shown to down-regulate ErbB-2 mRNA specifically in a previous study (40), to target ErbB-4 mRNA. In contrast to the ErbB-4 ribozyme, this ErbB-2 ribozyme did not alter the NRG1-α-induced IL-3 independence of ErbB-4-expressing 32D cells. These data suggest that Rz6 and Rz29 are functional ribozymes, and that the effects of these ErbB-4 ribozymes are highly specific to the ErbB-4 receptor mRNA. Rz29 exhibits a higher level of biological activity compared with Rz6. Rz21 apparently is a nonfunctional ribozyme in 32D cells. The inability of Rz21 to mediate the down-regulation of ErbB-4 may be due to several possibilities. For example, the target site may not be accessible intracellularly, or Rz21 may be unstable in 32D cells.

**Fig. 4. Regulation of receptor tyrosine phosphorylation by NRG1-α in 32D/E4 and 32D/E2 + E3 cells.** Five hundred μg of lysates from untreated or NRG1-α (100 ng/ml for 5 min) treated 32D transfectants (32D/wt, 32D/E2, 32D/E3, 32D/E4, and 32D/E2 + E3) were immunoprecipitated with anti-receptor antibodies (αE2, αE3, and αE4). 32D/E4/FGR cells (E1) were treated with 100 ng/ml of EGF for 5 min and immunoprecipitated with anti-EGFR antibody (αE1). +, lysates from EGF or NRG1-α-treated cells; –, lysates from untreated cells. The precipitates were then subjected to Western blotting with an anti-phosphotyrosine antibody (UBI). MW, molecular weight; IP, immunoprecipitation.

**Table 1 Effect of ErbB-4 ribozymes on the density of 32D/E4 cells in response to IL-3 starvation and HRG stimulations.**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Number of viable cells (×1000 cells/ml)</th>
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<tr>
<td></td>
<td>-IL-3</td>
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<tr>
<td>E4</td>
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<tr>
<td>E4/Vector</td>
<td>1</td>
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<tr>
<td>E4/Rz8</td>
<td>1.1</td>
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<tr>
<td>E4/Rz21</td>
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<tr>
<td>E4/Rz29</td>
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<tr>
<td>E4/ErbB-2 ribozyme</td>
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<tr>
<td>E4/E4/Rz6</td>
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<tr>
<td>E4/R29</td>
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<tr>
<td>E4/E4/R29</td>
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**Fig. 5. ErbB-4 ribozyme abolishes NRG1-α-induced mitogenesis.** 32D-transfected cells were plated at a density of 1 × 10^4 cells with or without IL-3 or with 100 ng/ml NRG1-α in the absence of IL-3. Two days after plating, the cells were labeled with [3H]thymidine for two hours. [3H]Thymidine incorporation was then analyzed by scintillation counting. wt, parental 32D cells; E4, 32D/E4 transfected cells. E4+/V, empty vector-transfected 32D/E4 cells. Ribozyme-transfected cells are indicated as Rz6, Rz21, and Rz29. Rz29 abolished the NRG1-α-induced IL-3-independent growth. All samples were prepared in triplicate. This assay was repeated three times. The SD was within 10%. □, IL-3; □, no IL-3; □, HRG.
type in ErbB-4 transfectants correlated with an increase in receptor tyrosine phosphorylation, the autophosphorylation of the receptors in these cells was examined by a kinase assay. Fig. 7 demonstrates that the level of ErbB-4 intrinsic tyrosine kinase activity in Rz29-transfected cells was markedly reduced, compared with control transfectants (32D/E4 and 32D/E4/Vector). Because ErbB-4 expression was down-regulated only 65% by Rz29, the cells still express ErbB-4 receptors. NRG1-α was therefore still able to induce the phosphorylation of the remaining ErbB-4 receptors. However, the level of phosphorylation was significantly lower than the 32D/E4 cells or the vector-transfected cells (32D/E4/Vector). Reduction of phosphorylation correlated with a reduction in expression of ErbB-4. Furthermore, these data also imply that although Rz29 is specifically cleaving its target mRNA, it does not affect the function of those receptors that are expressed. These intracellular experiments demonstrated that the decrease of ErbB-4 protein production, activation, and mRNA expression correlate with the ErbB-4 ribozyme catalytic activity.

**Effect of Down-Regulation of ErbB-4 Receptor in Human Breast Cancer Cells**

To investigate the biological and biochemical functions of ErbB-4 in human breast cancer, we expressed the ErbB-4 ribozymes in several ErbB-4-positive human breast cancer cell lines. One of the cell lines was T47D, derived from a breast carcinoma. The T47D cells express moderate levels of all of the presently known ErbB receptors. We transfected all three ribozymes (Rz6, Rz29, and Rz21), as well as the empty vector alone and G to A mutants of Rz29 and Rz6. The stably transfected clones were selected by G418. We observed a reduction in G418-resistant colony formation when the Rz6 and Rz29 constructs were transfected. This was evident especially in the Rz29 transfection, and it was extremely difficult to select the stably transfected clones, suggesting that down-regulation of ErbB-4 receptor in T47D cells may be lethal. We partially characterized the pooled population of the Rz6-transfected cells. We detected 70% down-regulation of the ErbB-4 receptor in these cells by FACS analysis (Fig. 8D), whereas no effect on the level of EGFR, ErbB-2, or ErbB-3 receptors was observed (Fig. 8, A–C). Ribozyme mediated down-regulation of ErbB-4 receptor expression in T47D/Rz6 cell was also confirmed by reduction of ErbB-4 mRNA level (Fig. 9). In addition, we observed that anchorage-independent colony formation was significantly reduced (65%) in the ribozyme Rz6-transfected cells (Fig. 10). Furthermore, the nonfunctional ribozyme (Rz21) and the G-to-A mutant
ribozymes have no effect on the level of ErbB-4 and no effect on cell proliferation, as well as the empty vector-transfected cells (Fig. 8, E and F). These preliminary data suggest that Rz6 is able to down-regulate the endogenous ErbB-4 receptor. The ErbB-4 receptor may therefore play a role in T47D cell proliferation.

**DISCUSSION**

In this study, we generated three specific hammerhead ribozymes targeted to ErbB-4 mRNA. We have demonstrated that these ErbB-4 ribozymes (Rz6, Rz21, and Rz29) effectively catalyze precise cleavage of ErbB-4 mRNA under physiological conditions in an extracellular system (Fig. 2). Furthermore, we demonstrated that these ribozymes do not cleave mRNA other EGFR family members, despite the high degree of sequence homology shared by these receptors. Point mutation of these ErbB-4 ribozymes in the catalytic domain resulted in loss of catalytic activity and failure to cleave ErbB-4 mRNA. These inactive ribozymes have identical binding arms to the active version but have a mutated catalytic domain. Thus, these mutated versions are capable of binding to the target sequence but are not able to cleave the target mRNA. Taken together, these control experiments demonstrate that the ErbB-4 ribozymes are highly specific for the ErbB-4 mRNA.

Using the 32D cell system to study the intracellular enzymatic activity of ErbB-4 ribozymes, we clearly demonstrated that the ribozymes are specific and effectively down-regulate the EGF receptor family members. In this system, one ErbB-4 ribozyme (Rz29) significantly reduced the ErbB-4 mRNA level and down-regulated ErbB-4 receptor expression (Fig. 6), thereby reversing the NRG1-α-induced IL-3-independent phenotype of 32D/E4 cells (Table 1). Rz6 partially down-regulated the expression of the ErbB-4 receptor and somewhat blocked the IL-3-independent phenotype. In contrast, Rz21 failed to down-regulate the ErbB-4 expression and inhibit the mitogenic response to NRG1-α treatment in 32D/ErbB-4 cells. It is clear from
these data that not all of the sites tested are equally amenable to intracellular ribozyme-mediated cleavage. This is in spite of the fact that ribozymes to all of the sites were shown to be catalytically active extracellular biochemical assays. RNA secondary structure or association with cellular proteins may affect target site accessibility. This demonstrates the need for an empirical determination of appropriate target sites. We therefore investigated the specificity and efficacy of these ribozymes in a well-defined cellular system. Two sets of experiments were conducted to control for ribozyme specificity and efficacy intracellularly. Because of the high level of homology between the EGF receptor family members, the intracellular specificity of ErbB-4 ribozymes was demonstrated using 32D cells that ectopically coexpress ErbB-2 and ErbB-3. None of the ErbB-4 ribozymes (Rz6, Rz21, and Rz29) had any effect on the level of ErbB-2 or ErbB-3 expression or the NRGl-a-induced IL-3-independent phenotype in these 32D derivative cells (Table 1). Moreover, an ErbB-2 ribozyme, shown previously to down-regulate the expression of ErbB-2 mRNA, failed to decrease ErbB-4 expression in 32D/ErbB-4 cells. The lack of down-regulation of ErbB-4 expression in these control experiments is evidence that these ErbB-4 ribozymes are highly specific. Furthermore, in the absence of NRGl-a, cells expressing these ribozymes remained strictly dependent on IL-3 for growth. In contrast, two ErbB-4 ribozymes (Rz29 and Rz6) decreased NRGl-a-induced, IL-3-independent proliferation. These phenomena indicate that only the ErbB-4 transcript is directly affected by these ribozymes. Although the ErbB-4 expression was reduced in Rz6- and Rz29-transfected 32D/E4 cells, the remaining ErbB-4 receptors in these cells were still phosphorylated in response to NRGl-a treatment (Fig. 7). This characteristic provides strong support for a cleavage-mediated mechanism of action for the ribozymes. Therefore, the constructed ErbB-4 Rz29 and Rz6 are biologically functional ribozymes and are highly specific for the targeted ErbB-4 mRNA in 32D cells.

To evaluate the effects of the down-regulation of ErbB-4 in an ErbB-4-positive human breast cancer cell line, Rz6 and Rz29 were transfected into T47D cells. We observed a reduction in G418-resistant colony formation in Rz6 transfection. However, we were unable to select clones after Rz29 transfection of T47D cells. These phenomena were not observed in the control ribozyme transfections. The low efficiency of Rz6- and Rz29-expressing, drug-selected clones are unlikely due to nonspecific effects, because all of the ribozymes were cloned into the same vector. One possibility is that if ErbB-4 plays a dominant role in T47D cell proliferation, a complete down-regulation of ErbB-4 may be lethal. Therefore, the inability to select Rz29 clones could be explained by a very efficient ErbB-4 down-regulation and may thus significantly inhibit cell proliferation; isolation of stably transfected T47D cells would be impossible. We were able to select the pooled population of the Rz6-transfected cells. Therefore, we selected Rz6-transfected T47D for further characterization. We observed that the ErbB-4 mRNA was significantly reduced by the ribozyme, and the ErbB-4 receptor expression was down-regulated by 70% in Rz6-transfected T47D cells. Down-regulation of the ErbB-4 receptor in T47D/Rz6 cells resulted in a reduction of colony formation in an anchorage-independent assay and in transfection efficiency, compared with vector- or Rz21-transfected cells. Furthermore, Rz6 only down-regulated ErbB-4 but not other ErbB-receptor family members. Reduction of colony formation suggests that ErbB-4 expression and mitogenic signaling may be essential for T47D cell survival. To confirm the role of ErbB-4 in cell proliferation, it will be important to extend this study to additional breast cancer cell lines, which express varying levels of ErbB family receptors. Further characterization of ErbB-4 ribozyme-transfected human breast cancer cells will be addressed in a future publication. Presently, we are also conducting these studies using an inducible promoter system. These preliminary findings suggest that a down-regulation of ErbB-4 expression by ErbB-4 ribozymes, as shown by FACS, diminished ErbB-4-mediated intracellular signaling. Because of heterodimerization between the family receptors, down-regulation of the ErbB-4 receptor may also be indirectly interrupting receptor signaling pathways initiated by other family members. This could result in diminished tumorigenicity in T47D cells. These results also show that our ribozyme is active in a human carcinoma cell line.

32D cells are strictly dependent upon IL-3 for survival and proliferation. However, NRGl-a was capable of stimulating its cognate receptors, coupling to cellular signaling pathways in 32D derivatives, and thereby abrogating IL-3 dependence of these cells. Using the ErbB-4 ribozymes in 32D cell system, we provide the first evidence that different threshold levels of ErbB-4 expression and activation correlate with different responses to NRGl-a stimulation. High levels of ErbB-4 expression, phosphorylation, and homodimerization are necessary for NRGl-a-stimulated IL-3-independent cell proliferation in the 32D/E4 cells. Low levels of ErbB-4 expression do allow NRGl-a-induced phosphorylation but are insufficient to couple receptor activation to cellular signaling, particularly in the case of Rz29-transfected 32D/E4 cells. In line with these observations, a recent study using Ba/F3 cell derivatives showed that NRGl-a failed to induce the IL-3-independent pathway in the ErbB-4 transfected cells (28). It is possible that the level of ErbB-4 expression in these Ba/F3/ErbB-4 cells is lower than that of our 32D/E4 cell line. We demonstrate that the IL-3-independent pathway appears to be very sensitive to the amount of ErbB-4 expression, as well as the tyrosine phosphorylation level. The ErbB-4 expression in Rz6-transfected cells was down-regulated by 45% and exhibited a weak response to NRGl-a. On the other hand, the Rz29-transfected cells, the ErbB-4
expression level of which was down-regulated by 65%, failed to respond to NRG1-α stimulation. NRG1-α was still able to induce ErbB-4 receptor phosphorylation in these cells, but the level of phosphorylation was much lower than in the 32/EA cells. This level of phosphorylation is not sufficient to stimulate the cellular response. These results also suggest that homodimers of ErbB-4 can transmit biological signals. This is consistent with a previous report that ErbB-4 homodimers constitute a functional NRG1-α receptor (23). NRG1-α can induce 32D/ErbB-2 + ErbB-3 cells to bypass the IL-3-dependent pathway, presumably due to transphosphorylation and cross-talk between the receptors through heterodimerization of ErbB-2 and ErbB-3. These results are consistent with previous studies concerning ErbB receptor transphosphorylation (34). Although ErbB-3 appears to be a defective tyrosine kinase receptor, it mediates biological signals. This is consistent with a previous report that expression of ErbB-4 in human breast cancer cells. Further implications of ErbB-4 expression in human breast cancer cells. Furthermore, our study supports the potential for using ribozymes as therapeutic agents for human breast cancer.

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ErbB-4 Ribozymes Abolish Neuregulin-induced Mitogenesis


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