ErbB-4 Ribozymes Abolish Neuregulin-induced Mitogenesis

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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 ribozyme 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 (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). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 12/2/97; accepted 6/2/98.

1 The abbreviations used are: EGFR, epidermal growth factor receptor; NRG, neuregulin; HRG, heregulin; IL, interleukin; FACS, fluorescence-activated cell sorter.

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3 We thank Dr. D. J. G. for providing the cDNA for human ErbB-4.

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Ribozyme-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 TTG-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- HCl (pH 8.0) and 20 mM MgCl2. Substrate and ribozyme transcripts were then mixed and incubated at 50°C for 30 min. Reaction products were analyzed on 6% urea 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 genetin (G418-sulfate; Life Technologies, Inc.).

Northern Blot Analysis. Total RNA from cell cultures was isolated using RNA solubilizer 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 Na3VO4. Remaining steps were performed on ice. Reconstituted HRG-β3 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-3; 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 Riese 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 (Ommias 3600; Imaging Products International, Inc., Charley, VA).

Mitogenic Assay. 32D transfected cells were plated at a density of 1 × 10⁶ cells with or without IL-3 supplement or supplemented with 100 ng/ml of NRG1-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 with β-scintillation counter.

In Vitro Kinase Assay. 32D/E4, 32D/E6 + V, and 32D/E4 + Rz29 cells were serum starved for 2 h before treatment with or without 100 µg/ml of NRG1-α. Cells then lysed in lysis buffer. Four hundred µg of total protein of each cell line was used to immunoprecipitate with anti-ErbB-4 antibody (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, 10 µg/ml 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...
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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: 518, 110 nucleotides; Rz21: 285, 337 nucleotides; Rz29: 322, 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.

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 transfecants 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-2 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-4-expressing 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.

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

Fig. 3. Growth assay. 32D cells were plated at a density of 1 × 10^4 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%. m^3^, IL-3; m, no IL-3; m, HRG.
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.

ErbB-4 Ribozyme Abolishes the NRG1-α Stimulation of Mitogenesis. To confirm the growth-inhibitory activity of the ErbB-4 ribozymes, a mitogenic assay to measure DNA synthesis was performed on ErbB-4Rz-transfected cells. As shown in Fig. 5, all of the 32D-transfected cells exhibited very low levels of \(^{3}H\)thymidine incorporation in the absence of IL-3. In contrast, all of the 32D-transfected cells exhibited high levels of \(^{3}H\)thymidine incorporation in the presence of IL-3, as expected. In the 32D/E4 control cells, NRG1-α stimulated high levels of \(^{3}H\)thymidine incorporation in the absence of IL-3, whereas the \(^{3}H\)thymidine incorporation was almost completely abolished in the Rz29-transfected cells. \(^{3}H\)Thymidine incorporation was significantly reduced in Rz6-transfected cells, but to a lesser extent than in Rz29-transfected cells. No significant changes in the Rz21-transfected cells were observed. These results were consistent with the growth assay.

ErbB-4Rz-mediated Down-Regulation of ErbB-4 Expression in 32D/ErbB-4 Cells. To evaluate the intracellular enzymatic cleavage activity of ErbB-4 ribozymes, the ribozyme transfecteds were examined for ErbB-4 mRNA levels by Northern blot analysis. Rz26- and Rz29-expressing cells exhibited significantly reduced ErbB-4 mRNA levels relative to control cells or to Rz21-expressing cells (data not shown). Thus, the abolishment of the NRG1-α-induced IL-3 independent biological effect correlates with reduction of ErbB-4 mRNA levels in these cells.

To further characterize the ribozyme effect, we quantitatively examined the ErbB-4 ribozyme-mediated down-regulation of ErbB-4 receptor expression in these ErbB-4Rz-transfected cells by FACS analysis. Consistent with Northern analysis, Rz29- and Rz6-transfected cells expressed significantly less cell surface ErbB-4 receptor relative to the 32D/E4 control cells (65 and 45% less ErbB-4, respectively; Fig. 6). No significant reduction of ErbB-4 expression was detected in Rz21-transfected cells. These data suggest that ErbB-4 Rz29 and Rz6 are biologically functional ribozymes.

Reduction of Autophosphorylation by ErbB-4 Ribozymes. To determine whether the NRG1-α-induced IL-3-independent pheno-
Fig. 6. Rz29 down-regulation of ErbB-4 expression in 32D/ErbB-4 cells. The levels of ErbB-4 in 32D/E4 and Rz29-transfected 32D/E4 cells were quantitatively measured by flow cytometry. Cells (1 x 10⁶) were harvested and stained with an anti-ErbB-4 monoclonal antibody in combination with fluorescence-labeled anti-mouse IgG antibody and analyzed by FACSscan. A. expression of ErbB-4 in vector-transfected cells (E4/V). Right-hand curves, specific staining; left-hand curves, nonspecific staining (primary antibody omitted); ordinate, relative cell number; abscissa, log fluorescence. B. Rz29 down-regulates ErbB-4 expression by 50%. Dotted-line curve, ErbB-4 expression in ErbB-4/V cells. Solid-line curve, ErbB-4 expression in Rz29-transfected cells. C. Rz21 has no effect on ErbB-4 expression. Dotted-line curve, ErbB-4 expression in ErbB-4/V cells. Solid-line curve, ErbB-4 expression in Rz21-transfected cells. D. Rz6 down-regulates ErbB-4 expression by 30%. Dotted-line curve, ErbB-4 expression in ErbB-4/V cells. Solid-line curve, ErbB-4 expression in Rz6-transfected cells.

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 EGF receptor 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 ErbB-4 ribozymes was demonstrated using 32D cells that ectopically coexpress ErbB-2 and ErbB-3. None of the ErbB-4 ribozymes (R26, R221, and R29) had any effect on the level of ErbB-2 or ErbB-3 expression or the NGF1-α-induced IL-3-independent phenotype in these 32D derivative cultures (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 NGF1-α, cells expressing these ribozymes remained strictly dependent on IL-3 for growth. In contrast, two ErbB-4 ribozymes (R29 and R26) decreased NGF1-α-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 R26- and R29-transfected 32D/E4 cells, the remaining ErbB-4 receptors in these cells were still phosphorylated in response to NGF1-α treatment (Fig. 7). This characteristic provides strong support for a cleavage-mediated mechanism of action for the ribozymes. Therefore, the constructed ErbB-4 R29 and R26 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, R26 and R29 were transfected into T47D cells. We observed a reduction in G418-resistant colony formation in R26 transfection. However, we were unable to select clones after R29 transfection of T47D cells. These phenomena were not observed in the control ribozyme transfections. The low efficiency of R26- and R29-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 Rz26-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 ribozyme, 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, NGF1-α 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 NGF1-α stimulation. High levels of ErbB-4 expression, phosphorylation, and homodimerization are necessary for NGF1-α-stimulated IL-3-independent cell proliferation in the 32D/E4 cells. Low levels of ErbB-4 expression do allow NGF1-α-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 NGF1-α 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 NGF1-α. On the other hand, the Rz29-transfected cells, the ErbB-4

Fig. 9. Northern blot analysis of ribozyme-mediated down-regulation of ErbB-4 mRNA in T47D/Rz6 cells. Total RNA was prepared from cultured cells (T47D/wt and T47D/Rz6). Gels were loaded with 20 μg of RNA per lane, transferred to nylon membranes, and hybridized with radiolabeled ErbB-4 probe. 18S RNA is shown as a loading control after the gel was stained with ethidium bromide.

Fig. 10. Anchorage-independent growth assay. The expression of ErbB-4 ribozyme in T47D cells (T47D/Rz6 pool clone) inhibits colony formation by more than 50%. A bottom layer of 0.1 ml Iscove’s modified Eagle’s medium 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 on a 0.8 ml top layer containing 0.4% Bacto Agar, and 5% FCS. All samples were prepared in triplicate. The cells were incubated for approximately 12 days at 37°C. Colonies larger than 60 μm were counted in a cell colony counter.
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expression level of which was down-regulated by 65%, failed to respond to NGFR-$\alpha$ stimulation. NGFR-$\alpha$ was still able to induce ErbB-4 receptor phosphorylation in these cells, but the level of phosphorylation was much lower in the 32/E4 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 NGFR-$\alpha$ receptor (23). NGFR-$\alpha$ 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 ErbB-4 ribozymes should provide specific target ErbB-4 mRNA for degradation extracellularly and intracellularly. These functional ErbB-4 ribozymes should provide important tools for delineating the biological and biochemical consequences 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.

ACKNOWLEDGMENTS

We thank Corinne Boulanger and Scott Brazinski for technical assistance.

REFERENCES

ErbB-4 Ribozymes Abolish Neuregulin-induced Mitogenesis


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