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TITLE: Inactivation of FGF Receptors by Targeting Ribozymes Against FGFR mRNAs and Their Effect on FGF Dependent In Vitro and In Vivo Breast Cancer Growth Phenotypes

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Acquired tamoxifen resistance is common in breast cancer patients with estrogen receptor positive (ER+) tumors. Growth factor signaling can provide ER+ breast cancer cells with alternative growth stimulus to that provided by activation of ER. In order to determine whether an individual FGF receptor (FGFR) or multiple receptors are responsible for conferring an alternate growth signaling pathway, we are using a ribozyme targeting strategy to selectively inactivate each of the receptors either singly or in combination. We have designed multiple ribozymes against each of the four receptors and tested their ability to cleave in vitro transcribed portions of FGFR RNA. These assays have indicated that at least one ribozyme against each receptor is able to efficiently cleave its target substrate in vitro. Next, we used transient transfection assays to determine the ability of each ribozyme to inhibit FGF-1 dependent phosphorylation of a cotransfected reporter encoding a Green Fluorescent Protein-MAPK fusion protein by Western blot analysis with phosphospecific antibodies. These studies indicate that ribozymes against individual FGFRs were not able to block phosphorylation of GFP-MAPK to the same extent as observed following cotransfection with a dominant negative FGFR.

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INTRODUCTION

The majority of patients with breast cancer possess tumors that are positive for the estrogen receptor (1). As a result, these patients are able to benefit from hormonal therapies involving antiestrogenic drugs such as tamoxifen. Unfortunately, these tumors eventually acquire resistance to tamoxifen as well as second line hormonal therapies. The exact cause of this resistance is not known, but interaction of the estrogen receptor with growth factor signaling pathways is thought to play at least a partial role. Previous studies in this lab have shown that inactivation of the Fibroblast Growth Factor Receptors (FGFRs) by over expression of a dominant negative FGFR results in abrogation of tumor cell proliferation under FGF dependent conditions (2). This dominant negative receptor was shown to heterodimerize with at least three of the four receptors and therefore was unable to determine which specific FGF receptor or combination of receptors is responsible for the FGF dependent growth factor signaling. In an effort to determine whether an individual FGF receptor family member or multiple receptors are responsible for conferring an alternate growth signaling pathway, we are utilizing a ribozyme targeting strategy to selectively inactivate each of the receptors either singly or in combination.

BODY

For the design of the ribozymes, we first located the appropriate FGFR sequences from Genbank and downloaded these via the internet. Due to the fact that there are various alternative splice forms for each receptor and that these isoforms are often cell type specific, we chose those isoforms which have been shown to be present in cells of epithelial origin. In addition, we performed a sequence search for similarity to verify that the sequences were unique. We then utilized the MFOLD program that is part of the Genetics Computer Group (GCG) software package to generate graphical models in order to select potential target sites against each of the FGFR. This program utilizes free energy minimization methods developed by Zucker et. al. (3) to predict optimal and suboptimal 3-dimensional structures of RNA molecules. Using certain target site selection rules as described in the literature (4), we chose three target sites against each FGFR (Figures 1 & 2). Multiple target sites were chosen in order to increase the chance of selecting a highly efficient ribozyme against a particular FGFR. Once target sites were chosen, sense and antisense strands were synthesized (Genosys) for each ribozyme. Each pair of desalted and gel purified oligonucleotides were then annealed, phosphorylated, and ligated into the pcDNA3 expression vector (Invitrogen) utilizing NotI and HindIII overhangs. All constructs were confirmed by restriction digest analysis and dideoxysequencing in order to verify the presence and orientation of the correct insert.

After design and synthesis of the ribozymes, we next tested the ability of the ribozymes to cleave in vitro transcribed FGFR target RNA. A T7 or Sp6 promoter was used to generate radio-labeled sense strand RNA substrate from cDNA template spanning regions of the appropriate FGF receptor that contained the target sequence. This cDNA was generated by RT-PCR of total RNA from MCF-7 cells in the presence of appropriate primers that contained appended 5' and 3' restriction enzyme cleavage sites to facilitate subsequent cloning of the gel purified and digested PCR products into the pcDNA3 expression vector. We initially experienced some difficulty in the cloning of the target fragments into this vector using the NotI and HindIII restriction sites. To overcome this, we utilized a T/A cloning strategy using the pCRII vector (Invitrogen). For these assays, a T7 promoter upstream of the ribozyme inserts was used to generate cold in vitro run off transcripts of single stranded RNA containing the antisense ribozyme. Single stranded p-32 labeled FGFR substrate transcripts less than 1000 nucleotides in length were mixed with ribozymes at molar ratios of 1:1, 1:10, and 1:100, and then incubated at 37°C in a 50µl reaction mixture that contained an RNAse inhibitor, 20mM $MgCl_2$, and 50mM EDTA. 10µl aliquots were removed at 5', 15', 1hr, 2hr, and 4hr and were run on denaturing polyacrylamide gels (BioRad). Controls for these assays included FGFR incubated without ribozyme in parallel and an inactive ribozyme. Results from these assays indicated that at least two of the three ribozymes against each receptor was able to specifically and efficiently cleave its target substrate in vitro (Figure 3).

Due to the fact that there is no clear correlation between in vitro and in vivo ribozyme activity (5), we next wanted to verify the effects of ribozyme expression on FGF dependent phenotypes using short-term colony forming assays. In initial experiments, ML-20 cells were cotransfected with a CMV promoter directing blasticidin

S resistance and either a control vector or a CMV driven ribozyme expression vector. Cells were stripped of estrogen in Improved Minimal Essential Media containing 5% Charcoal-stripped Fetal Bovine Serum (CFS) with four media changes over 48 hours. Cells were then trypsanized and plated at $1X10^4$ cells in 60mm dishes. Cells were transfected using Lipofectamine (Gibco) according to manufacturer's instructions. Immediately after transfection, cells were placed in phenol red free (PRF) IMEM containing 5% FBS and appropriate conditions. Cells received either 5%FBS only, or 5% FBS supplemented with either ICI 182-780 (ICI) at 10^{-7} M, ICI + FGF-1 at 20ng/mL, or ICI + estrogen at 10^{-8} M. All conditions received heparin sulphate at a final concentration of 50µg/mL. If the catalytically active ribozymes were capable of inhibiting FGF dependent growth phenotypes, we expected to see a reduction in colony formation compared to inactive controls and vector controls in media containing FBS + ICI. Overall growth of ML-20 cells in these assays was relatively weak, however, and therefore detection of a reduction in number of colonies formed was not possible and no conclusions could be drawn. Similar results were obtained with the Clone 18 cell line and in similar anchorage independent soft agar colony forming assays. To address this issue, we have begun working with the SW-13 adrenal cortex carcinoma cell line that expresses FGF cell surface receptors and has shown a more robust growth phenotype in these types of assays.

In order to establish in vivo ribozyme activity, we next tested the effects of ribozyme expression on MAPK phosphorylation using transiently transfected ML-20 cells. Studies in our lab have shown that a green fluorescent protein-tagged (GFP) map kinase (MAPK) protein can be used to distinguish between endogenous ERK1/2 and transiently transfected GFP-MAPK. Using this assay, we tested the ribozymes for their ability to abrogate FGF stimulated phosphorylation of the GFP- MAPK fusion protein. 100 mm dishes containing $1X10^6$ cells in 5% FBS-PRF-IMEM were cotransfected with CMV driven expression vectors encoding the ribozymes and the GFP-MAPK and the effects of ribozyme expression on MAPK phosphorylation were evaluated by Western blot analysis. 24 hours after plating, cells were transiently transfected with Lipofectamine (Gibco) according to manufacturer's instructions and allowed to incubate at 37°C in 5% CO_2 for 24 hours. Cells were then stripped in four changes of 5% Charcoal-stripped fetal Calf Serum (CCS) over a 24 hour period and then maintained in serum free IMEM for an additional 24 hours. Cells then received no treatment or were treated with 20ng/mL FGF-1 for 30 minutes. Cells were lysed using Lysis Buffer (New England Biolabs) and the concentration of protein in each lysate was determined using the BCA Protein Assay Kit (Pierce). 30µg of protein from each lysate in loading buffer was boiled for 5 minutes, electrophoresed on a 10% SDS-PAGE precast Criterion™ gel (BioRad), and electrotransfered to a nitrocellulose membrane. The membranes were stained using a Phospho-plus MAPK p44/42 Antibody Kit (Cell Signaling) according to the manufacturer's instructions for the presence of phosphorylated and non-phosphorylated forms of MAPK 1 and 2, respectively. The immunochemical staining was detected using enhanced chemiluminescence (Supersignal, Pierce). Results from these experiments indicated that ribozymes targeted against individual receptors were not able to reduce the level of GFP-MAPK phosphorylation to the same levels as that of a dominant negative FGFR (Figure 4).

Key Research Accomplishments

- Designed and synthesized 3 ribozymes against each of the four FGFRs, plus one inactive ribozyme control
- Shown that 11 of the 12 ribozymes exhibit catalytic activity versus in vitro transcribed target transcripts
- Presented data at the American Association for Cancer Research 2002 annual convention
- Shown that transient expression of ribozymes directed against individual FGF receptors is not as effective at reducing GFP-MAPK phosphorylation as a dominant negative FGFR as determined by western-blotting

Reportable outcomes

Norman Estes, Jaideep Thottassery, and Francis G. Kern. Inactivation of FGF Receptors by Targeting Ribozymes against FGFR mRNAs and Their Effect on FGF Dependent *in vitro* and *in vivo* Breast Cancer Growth Phenotypes. Proceedings of the 93rd Annual Meeting of the American Association for Cancer Research. April 6-10, 2002, San Francisco, California, p. 727.

Conclusions

At the end of the first year of this study, we have completed the design and synthesis of multiple ribozyme against each of the FGF receptors. In addition, we have demonstrated that at least two ribozymes against each FGF receptor are able to efficiently cleave in vitro transcribed FGFR target substrate utilizing in vitro cleavage assays. Initially, we experienced some difficulty in verifying ribozyme activity in ML-20 cells using transiently transfected cells in colony forming assays. In general, cell growth was not robust in these assays, making determination of any potential effects of ribozyme expression difficult. In order to address this issue we are currently utilizing the SW-13 cell line, which expresses cell surface FGF receptors and responds to exogenous acidic FGF by forming colonies in soft agar. To further address the in vivo effects of ribozyme expression against single FGF receptors, we have examined the effect of ribozyme expression on the phosphorylation of a GFP-MAPK fusion protein. These studies indicated that ribozyme expression against individual receptors was not as effective at preventing GFP-MAPK phosphorylation as a dominant negative FGF receptor.

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Appendix

Figures and Legends

Abbreviations

CCS	charcoal stripped fetal calf serum
EDTA	ethylenediamine-tetraacetic acid
ER	estrogen receptor
ER+	estrogen receptor positive
ERK1/2	extracellular signal regulated kinase 1/2
FGF	fibroblastic growth factor
FGFR	fibroblastic growth factor receptor
FBS	fetal bovine serum
GCG	genetics computer group
GFP	green fluorescent protein
IMEM	Improved Minimal Essential Medium
MAPK	mitogen activated protein kinase
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid

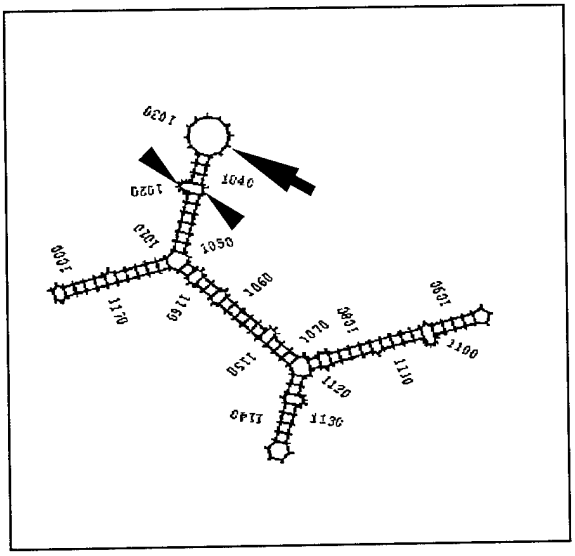
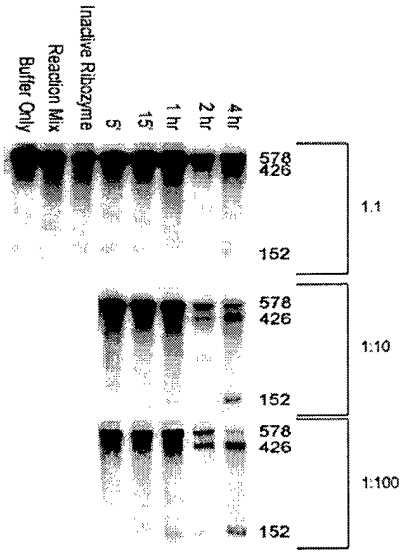


Figure 1 Partial stem-loop structure of the FGFR-1 mRNA. The arrows outline a typical target site with the large arrow indicating the cleavage point and the smaller arrows indicating the ends of the flanking regions

Receptor	Ribozyme	Fragment Length	Band A	Band B	Cleavage nt	Target Region
FGFR1A	Rbz1.1	605	153	452	587	Ig II
FGFR1B	Rbz1.2	578	313	265	1135	Ig III
FGFR1B	Rbz1.3	578	426	152	1248	TM
FGFR2	Rbz2.1	605	202	403	1136	Ig II
FGFR2	Rbz2.2	605	272	333	1206	Ig II
FGFR2	Rbz2.3	641	473	168	1371	Ig III
FGFR3A	Rbz3.1	382	182	200	493	Ig II
FGFR3B	Rbz3.2	551	190	361	1026	Ig III*
FGFR3B	Rbz3.3	551	351	200	1187	TM
FGFR4	Rbz4.1	631	183	448	720	Ig II
FGFR4	Rbz4.2	631	510	121	1047	Ig III
FGFR4	Rbz4.3	631	541	90	1078	Ig III

Figure 2 Fragments for *in vitro* cleavage assays and ribozymes designed against each receptor. Also shown is the length of each receptor target sequence, the fragment lengths resulting from cleavage of the target by a ribozyme designed against it, and the region within the FGFR to which the ribozyme is targeted. rbz3.2 was designed against a target site within the C-terminal half of the 3rd Ig loop of FGFR3 (FGFR3 IIIb isoform).

A



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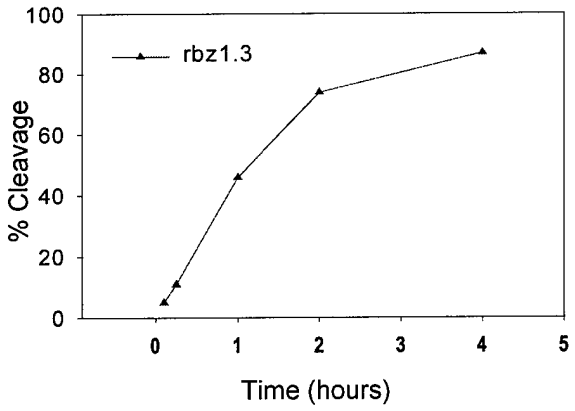

Figure 3 *In vitro* runoff transcripts were generated utilizing either T7 or Sp6 promoter sites. ^{32}P labeled target substrate and cold ribozymes were mixed at molar ratios of 1:1, 1:10, or 1:100 in a reaction mixture containing 20 mM MgCl. Aliquots were removed at 5', 15', 1 hr., 2 hr., and 4 hr. and cleavage products were viewed on a denaturing polyacrylamide gel.

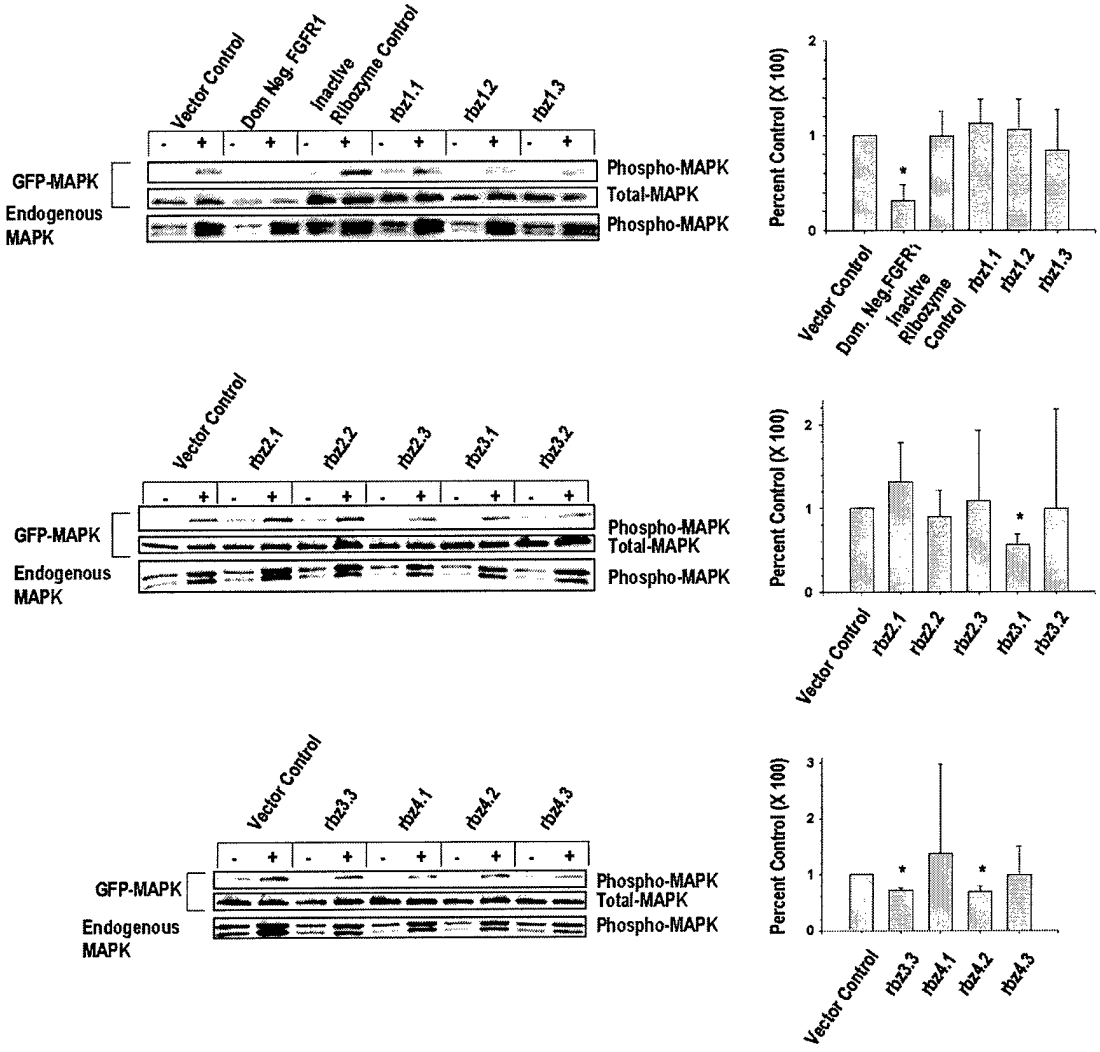

Figure 4 Western Blot analysis of ML-20 breast cancer cells transiently cotransfected with a Green Fluorescent Protein-MAPK fusion protein and CMV driven ribozymes directed against individual FGFRs. ML-20 cells were stripped of estrogen in 5% CCS-PRF-IMEM, incubated overnight in serum free PRF-IMEM, and incubated with -/+ 20 ng/mL FGF-1 for 30 minutes. 30µg of total cell lysate was loaded into each well of a 10% SDS-PAGE for immunoblotting with phospho-MAPK and MAPK antibodies. Quantitation was performed by densitometry on an Alpha Innotech MultiImagerTM LightCabinet and ChemiImager software. Vector control levels were arbitrarily set to 1. Samples were normalized to total GFP MAPK protein levels and the relative level of FGF-1 induced GFP-MAPK phosphorylation are expressed as a percentage of the vector control. Results are the mean (±SD) from 3 independent experiments. The asterisks indicate significant difference as determined by a t-test with $p < 0.05$.