Hammerhead Ribozyme-mediated Inactivation of Mutant RET in Medullary Thyroid Carcinoma

Ranjani Parthasarathy, Gilbert J. Cote, and Robert F. Gagel

Section of Endocrine Neoplasia and Hormonal Disorders, Department of Internal Medicine Specialties, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030

Abstract

Activating mutations of the RET proto-oncogene cause hereditary medullary thyroid carcinoma. To examine whether selective inactivation of mutant RET could prevent transformation, a hammerhead ribozyme was designed to cleave RET mRNA containing a transforming mutation of codon 634 TGC → TAC (Cys634Tyr). In vitro RNA cleavage assay demonstrated that the ribozyme selectively cleaved RET RNA with a Cys634Tyr but not Cys634Arg or the normal sequence. Expression of ribozyme in NIH/3T3 cells prevented RET-mediated colony formation in soft agar. This inhibition required catalytically active ribozyme and was specific for the TAC mutation. Therefore, ribozymes designed to selectively target mutant RET RNA may provide an effective therapeutic in the treatment of this syndrome.

Introduction

MEN2A, MEN 2B, and familial medullary thyroid carcinoma are autosomal dominant genetic cancer syndromes that affect several endocrine glands (1). MEN 2A is defined by the presence of MTC, pheochromocytoma, and hyperparathyroidism (1). MEN 2B is characterized by MTC, pheochromocytoma, skeletal abnormalities, ganglieneuromas of the intestinal tract, and mucosal neuromas (1). These syndromes are associated with germ-line mutations in one of the alleles of the RET proto-oncogene, a tyrosine kinase receptor. The majority of MEN 2A mutations (>95%) are specific to one of five codons in the extracellular cysteine-rich region of RET (Fig. 1; Ref. 2). The MEN 2B phenotype is associated with a single missense mutation, a T to C transition in the tyrosine kinase domain of RET that converts the methionine at codon 918 to a threonine (3). Metastatic MTC causes death in ~50% of patients (4). Early detection of gene carriers and surgical removal of the thyroid presently offers the only chance of a cure. Treatment modalities for patients with advanced metastatic disease are few, and none is curative. We conceptualized that a strategy to inactivate mutant RET in established tumors might remove the major stimulus to transformation. However, a normal receptor is known to play a significant role in several tissues and in development (5). Therefore, we designed a hammerhead ribozyme to specifically inactivate mutant RET, while sparing the normal receptor, and have examined the use of this ribozyme in a model system of transformation.

Materials and Methods

Cell Lines. The NIH/3T3 cell line (generously provided by Kapil Mehta, Department of Bioimmunotherapy, M. D. Anderson Cancer Center) was routinely maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated FCS (Life Technologies). Stably transfected cell lines were obtained by selection with Genetin (Life Technologies) following transfection of plasmid (1–1.5 μg) using a cationic lipid (Dosper; Boehringer Mannheim, Indianapolis, IN). Resistant cells were selected by treatment with 600 μg/ml of Genetin for 12 days and then a maintenance dose of 200 μg/ml.

Plasmid Constructs. Plasmids encoding normal (Cys634) or mutant RET (Cys634Arg, Met918Thr) in the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA) were kindly provided by Dr. Sissy Jhiang at the Ohio State University (6). Introduction of the codon 634 TAC→TAC (Cys634Tyr) mutation was performed with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and required subcloning of a KpnI fragment of RET into pBluescript II KS (Stratagene) to reduce plasmid size. The Cys634Tyr mutation was introduced using the primers 5'-GCAGCGTGACTTACGGGTCGCGTCGTA-3' (forward) and 5'-TCACCGTGTCGCTACGCAGCGTCCGCGTTACCA-3' (reverse). Mutant clones were identified by the gain of the RsaI restriction site and ligated back to the mammalian expression vectors pcDNA3 (Invitrogen) for creation of stable cell lines.

Creation of inserts for the active and control ribozyme was performed by PCR using overlapping oligonucleotide primers. The active ribozyme used primers 5'-GCTAGCTGGCTGATGGTAGGCGGTTG-3' (forward) and 5'-CGAGGACTTCTTGCTGCAAGAGTCA-3' (reverse). For the control ribozyme, the forward primer was substituted with 5'-GCTAGCTGGCTGATGGTAGGCGGTTG-3', which mutates a functionally indispensable base in the catalytic core. The PCR products were then individually cloned into the vector pCR3 using the TA cloning kit (Invitrogen). All plasmid constructs were sequenced to confirm the mutagenesis.

In Vitro Cleavage Assay. Ribozymes and RNA substrates were synthesized by standard in vitro transcription reaction at 37°C for 1 h using T7 polymerase (Life Technologies). For RNA substrates, the transcription reactions incorporated [32P]ATP. The active and control ribozyme plasmids were used to generate in vitro transcription templates by PCR using primers 5'-TAATACGACTCATATAGGG-3' (forward) and 5'-TCACCGTGACTTACGGGTCGCGTCGTA-3' (reverse). Creation of template for the normal and mutant RET was performed by PCR using overlapping oligonucleotide primers. Template for normal RET used primers: 5'-TAATACGACTCATATAGGG-3' (forward) and 5'-CGAGGACTTCTTGCTGCAAGAGTCA-3' (reverse). For the TAC mutant RET template, the reverse primer was substituted with 5'-TCACCGTGACTTACGGGTCGCGTCG-3'. Ribozyme-catalyzed cleavage reactions were performed as follows. The ribozyme (active or catalytically inactive) and labeled substrates were resuspended in assay buffer (50 mM Tris, 100 mM KCl), denatured at 95°C, and gradually cooled to the reaction temperature (37–42°C), and the reaction was initiated by the addition of MgCl2 (1–10 mM). The reaction was terminated at the indicated times by the addition of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF). The cleavage products were then analyzed by denaturing electrophoresis on 20% polyacrylamide gel containing 8M urea. Samples incubated without active ribozyme served as controls for nontarget degradation of the RNA substrates.

Anchorage-independent Growth Assays. NIH/3T3 cells stably expressing RET or ribozyme were transiently transfected with the indicated constructs.

Received 4/21/99; accepted 7/1/99.

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1 This work was supported by the John Ball Foundation (R. F. G.) and an Institutional PRS Award (G. J. C.).
2 To whom requests for reprints should be addressed, at M.D. Anderson Cancer Center, Department of Internal Medicine Specialties, Box 047, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-6517; Fax: (713) 794-1818; E-mail: rgagel@mdanderson.org.
3 The abbreviations used are: MEN, multiple endocrine neoplasia; MTC, medullary thyroid carcinoma; RT-PCR, reverse transcription-polymerase chain reaction.
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Fig. 1. Schematic representation of the c-RET mRNA and the active ribozyme designed to target the mutation at codon 634. Upper sequence, specific region of the RET mRNA targeted for hybridization by the ribozyme. A control inactive ribozyme was also made by the mutation of a functionally indispensable base (G) in the catalytic core. Arrowhead, cleavage site.

Fig. 2. Assay of ribozyme cleavage activity. Cleavage reaction for TAC RNA substrate (left gel) or TGC RNA substrate (middle gel) by ribozyme. Right gel, cleavage reaction for RNA substrate containing mutant (TAC) sequence by control ribozyme. Reaction time courses were performed as described in “Materials and Methods.”

Results and Discussion

Ribozyme Design. The aim of this study was to determine whether a hammerhead ribozyme could be designed to specifically target and cleave mutant RET RNA while leaving the normal mRNA uncleaved. This class of ribozymes function by hybridizing to the target RNA via Watson-Crick base pairing to create a catalytic domain that is then able to cleave the target RNA by a trans-esterification reaction. There are minimal but specific sequence requirements at the cleavage site. Mutational analysis has revealed that optimal cleavage occurs after the sequence NUH, with N being any nucleotide and H being A, C, or U. In general, targets containing GUC, GUA, GUU, CUC, and UUC are well cleaved, and targets containing a G in the third position are not (7). Therefore, cleavage specificity can be attained by the presence or absence of a single G nucleotide. We chose to use this nucleotide specificity in designing a ribozyme to cleave mutant RET mRNA. The ribozyme targets a commonly found mutation at RET codon 634 that changes a normal cysteine (TGC) to a tyrosine (TAC), thereby creating a cleavage site in the mRNA (GUG→GUAA).

In Vitro Cleavage of Mutant RET RNA by Ribozyme. The activity and specificity of ribozyme-mediated cleavage was examined by in vitro assay. The nonstandard structure of the ribozyme used required optimization of cleavage temperature and MgCl₂ concentration (data not shown; Refs. 8 and 9). The ribozyme was observed to cleave a radiolabeled UAC mutant RNA (tyrosine mutation of RET) substrate in a time-dependent manner, with cleavage seen as early as 2 h and continuing up to 24 h (Fig. 2). Under identical reaction conditions, no cleavage was observed with the ribozyme when incubated with a normal UGC RET RNA substrate for up to 24 h (Fig. 2). As expected, a control ribozyme containing an inactivating mutation of the catalytic core also failed to demonstrate cleavage of mutant RNA substrate (Fig. 2). Therefore, these in vitro results indicate that the ribozyme was active and capable of discriminating between normal and mutant RNA substrates.

Ribozyme-mediated Reduction in RET-dependent NIH/3T3 Colony Formation. Previous studies have established that mutant forms of the RET gene (both MEN 2A and 2B), when introduced into NIH/3T3 cells, are highly clonogenic in the soft-agar assay (6, 10). The oncogenic potential is limited to these mutants because normal RET is incapable of colony formation. Therefore, inhibition of mutant RET expression would be expected to prevent colony formation. To test the in vivo action of our ribozyme, a construct was created to overexpress RET mRNA containing the codon 634 TGC→TAC mutation. NIH/3T3 cells were transfected with the mutant TAC-RET construct and then selected by G418 for stable expression of the mutant RET protein. These cells were then transiently transfected with constructs expressing either the active ribozyme, control ribozyme, or vector alone. In repeated experiments, we found that when TAC-RET-expressing cells were transiently transfected with the active ribozyme, there was a significant reduction (83%, P < 0.001) in colony formation compared with cells transfected with empty vector (Fig. 3A). When the same cells were transiently transfected with the control ribozyme, there was only a small reduction in colony formation that...
was not statistically significant (−10%, \( P > 0.05 \); Fig. 3A). These differences in inhibition of colony formation are consistent with a mechanism whereby the ribozyme is acting to reduce the expression of mutant RET RNA through a cleavage-mediated pathway rather than an antisense mechanism. To examine this more directly, we performed RT-PCR analysis on RNA isolated from cells stably expressing TAC-RET following transient transfection with the constructs described above (Fig. 3B). Transient transfection of the construct expressing the active ribozyme caused a reproducible reduction in RET mRNA when compared with cells transfected with vector (60% ± 4 SD in three experiments; Fig. 3B). Only a modest reduction in RET mRNA was observed in cells expressing control ribozyme (18% ± 15 SD; Fig. 3B). To provide additional support for a ribozyme-mediated cleavage, an analogous set of experiments was performed using NIH/3T3 cells stably expressing the active ribozyme or the control ribozyme. In these experiments, overexpression of the active ribozyme was found to prevent colony formation when cells were transiently transfected with TAC-RET-expressing plasmids (88% reduction in colony formation; Fig. 3C). Inhibition of colony formation was specific with cells expressing the active ribozyme, because the stable expression of the control ribozyme was associated with antisense activity showing a 27% reduction in colony number (\( P > 0.05 \); Fig. 3C). Therefore, overexpression of the active form of the ribozyme provides a protective effect against mutant RET-mediated transformation.

**In Vivo Specificity of Ribozyme.** The experiments above reveal the potential for ribozyme-mediated inhibition of RET-mediated transformation but do not address the question of ribozyme specificity. Furthermore, because expression of normal RET does not result in colony formation, it is not possible to directly assess the activity of the ribozyme on normal RET RNA in intact cells. To examine the question of *in vivo* specificity, we created NIH/3T3 cells that stably express two different RET (MEN 2) mutations previously described to cause colony formation (6). The first construct contains a different MEN 2A mutation, codon 634 TGC \( \rightarrow \) CGC. This construct lacks the NUH consensus sequence required for ribozyme cleavage. As expected, there was no significant difference in colony formation between cells transiently transfected with empty or ribozyme-expressing vector (Fig. 4). These results clearly demonstrate that the ribozyme...
action is sequence specific and provide additional support for a cleavage-mediated mechanism. However, the results obtained with the CGC-RET construct do not address the question of whether normal RET RNA is cleaved by the ribozyme. The second construct containing a MEN 2B mutation (codon 918, ATG→ACG) also induces colony formation in soft agar but leaves the normal codon 634 TGC unchanged (6). Therefore, if the ribozyme were capable of cleavage at the normal codon 634, transformation mediated through the codon 918 mutation would be prevented. This is not the case. Active ribozyme had no effect on 918 RET-mediated colony formation, indicating that there was no cleavage at the normal codon 634 (Fig. 4).

These studies provide in vitro and in vivo evidence that a ribozyme can be specifically targeted to a RET RNA containing a single nucleotide change. Furthermore, expression of active ribozyme is capable of preventing RET-mediated transformation of NIH/3T3 cells. Together the observations suggest that a ribozyme-based approach may provide a useful strategy for selective inactivation of mutant RET expressed in hereditary or sporadic medullary thyroid carcinoma. The current strategy for management of MEN is to identify gene carriers at an early age and perform a thyroidectomy prior to development of metastatic disease. Prompt thyroidectomy is also recommended in patients with sporadic disease. But few treatment modalities are available for patients who present with advanced metastatic disease and chemotherapy has proven to be ineffective. Targeted inactivation of the specific mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. Nature (Lond.), 365: 458–460, 1993.


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