Optimizing the Cell Efficacy of Synthetic Ribozymes

SITE SELECTION AND CHEMICAL MODIFICATIONS OF RIBOZYMES TARGETING THE PROTO-ONCOGENE c-myb

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Expression of the proto-oncogene c-myb is necessary for proliferation of vascular smooth muscle cells. We have developed synthetic hammerhead ribozymes that recognize and cleave c-myb RNA, thereby inhibiting cell proliferation. Herein, we describe a method for the selection of hammerhead ribozyme cleavage sites and optimization of chemical modifications that maximize cell efficacy. In vitro assays were used to determine the relative accessibility of the ribozyme target sites for binding and cleavage. Several ribozymes thus identified showed efficacy in inhibiting smooth muscle cell proliferation relative to catalytically inactive controls. A combination of modifications including several phosphorothioate linkages at the 5′-end of the ribozyme and an extensively modified catalytic core resulted in substantially increased cell efficacy. A variety of different 2′-modifications at positions U4 and U7 that confer nuclease resistance gave comparable levels of cell efficacy. The lengths of the ribozyme binding arms were varied; optimal cell efficacy was observed with relatively short sequences (13–15 total nucleotides). These synthetic ribozymes have potential as therapeutics for hyperproliferative disorders such as restenosis and cancer. The chemical motifs that give optimal ribozyme activity in smooth muscle cell assays may be applicable to other cell types and other molecular targets.

Since the discovery that certain naturally occurring RNA motifs were capable of catalytically cleaving other RNA molecules in a sequence-specific manner, extensive studies have defined the sequence and structural characteristics that control the in vitro specificity and kinetics of these RNA enzymes or ribozymes (1–4). Ribozymes have a broad range of potential in vivo applications. These include the use of ribozymes as research tools for probing molecular mechanisms, the use of ribozymes to genetically engineer crops, and the use of ribozymes as therapeutics for human or animal diseases. Each of these applications requires that a ribozyme function efficiently within the intracellular environment. The sequence and structural features that promote optimal intracellular activity of ribozymes are currently under study.

Several factors are likely to contribute to the intracellular efficacy of a ribozyme. A ribozyme must colocalize with its molecular target in the appropriate cellular compartment and must be present at sufficiently high concentration to promote hybridization. In addition, its catalytic cleavage rate must be fast enough, and its half-life must be long enough to allow cleavage of a substantial fraction of the target mRNA population. Finally, the cleavage site in the target mRNA must be accessible to ribozyme binding. When the ribozyme is made synthetically, a variety of modifications can be introduced to increase its half-life within the cell, to change its target sequence binding affinity, and possibly also to alter its intracellular trafficking properties. In this study, we have used chemically synthesized hammerhead ribozymes targeting the proto-oncogene c-myb to study different chemical modifications and sequence changes that affect cell efficacy. Expression of c-myb is necessary for cell-cycle progression in vascular smooth muscle cells (5, 6). Therefore, we have used proliferation of rat aortic smooth muscle cells as a measure of the efficacy of the ribozymes targeting c-myb (7).

Unmodified RNA is subject to rapid nuclease degradation upon exogenous delivery to cells or tissues. For example, the half-life of an all RNA hammerhead ribozyme in human serum is less than 0.1 min (8). In the literature there are several reports of exogenously delivered synthetic ribozymes showing efficacy in cell culture (9–12). Often, these studies have utilized DNA/RNA chimeric ribozymes to enhance resistance to exonucleases, leaving large regions of unmodified RNA susceptible to endonucleolytic degradation. Extensive modification of the hammerhead ribozyme motif can give dramatic enhancement of the ribozyme half-life in biological fluids (8, 13). Modifications of this type have been demonstrated to give efficiency in cell culture (7) and in vivo (13, 14).

We have previously described dose-dependent inhibition of smooth muscle cell proliferation by select ribozymes targeting c-myb. Little inhibition was observed with catalytically inactive control RNA molecules. The active ribozymes reduced the level of the target c-myb RNA (7). Thus, the smooth muscle cell assay is a suitable means of measuring ribozyme efficacy in cells. Here, we report on a systematic method for determining accessible ribozyme target sites and for determining the optimal hammerhead ribozyme arm length required for cell efficacy. In addition, we explore the effect of chemical modifications such as those reported by Beigelman et al. (8) on cell efficacy. We have thereby identified chemical motifs that maximize the potency and specificity of synthetic ribozymes delivered exogenously to cells in culture.

MATERIALS AND METHODS

Ribozyme Synthesis and Sequences—Ribozymes were synthesized and purified as described (15–17). The sequences and modifications of all of the active ribozymes used in this study are shown in Fig. 1. The cleavage site numbering is based on the human DNA sequence numbering (Genbank accession number X52125; transcription starts at...
nucleotide 198). The inactive ribozymes contain identical binding arms and catalytic modifications except that positions G5 and A14 in the catalytic core were changed to 2'-O-methyl uridine, thereby eliminating catalytic activity while maintaining nuclease resistance. The catalytic cleavage activity of all of the ribozymes was confirmed on a charted short substrate by standard methods; inactive ribozymes did not show detectable cleavage activity (data not shown).

Template RNA Transcription—A murine c-myb CDNA clone was obtained from Dr. Prema Kumar Reddy. Full-length c-myb RNA was prepared by T7 transcription. Reactions contained 40 mM Tris, pH 8.3, 10 mM MgCl₂, 10 mM NaCl, 10 mM dithiothreitol, 4 mM spermidine, 250 μM each rATP, rGTP, and rUTP, and 50 μM rCTP, 40 units of RNase inhibitor (Boehringer Mannheim), 80 μCi of a-CTP (40 μCi/μl; DuPont NEN), and 20 units of T7 Polymerase (United States Biochemical Corp.) in a volume of 20 μl and were incubated at 37°C for 2 h. The resulting internally labeled transcripts were purified over a G50 spin column (Pharmacia Biotech Inc.).

In Vitro RNase H Cleavage and Ribozyme Cleavage Reactions—15-nucleotide-long antisense DNA oligonucleotides were designed to anneal to potential hammerhead ribozyme binding sites in murine c-myb. 4–10 ng of internally labeled murine c-myb transcript was incubated with 10 μM oligonucleotide plus 0.8 units of RNase H (Life Technologies, Inc.) in 20 mM Tris, pH 7.9, 100 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA, 0.1 mM dithiothreitol at 37°C for 60 min. For the ribozyme cleavage assay, 1.0 μM ribozyme was incubated with 4–10 ng internally labeled substrate in 75 mM Tris, pH 8.0, and 10 mM MgCl₂ at 37°C for 1 h. Reactions were stopped by addition of formamide gel-loading buffer (95% formamide, 0.1% bromphenol blue, 0.1% xylene cyanol, 20 mM EDTA) and electrophoresed on a 6% denaturing acrylamide gel. Gels were dried and quantified on a phosphorimager.

Cell Culture—Rat aortic smooth muscle cells were isolated from aorta tissue explants from 69–84-day-old female Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.) and assayed through passage six. Rat aortic smooth muscle cells were grown in Dulbecco's modified Eagle's medium supplemented with non-essential amino acids (0.1 mM of each amino acid), 0.1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 20 mM Hepes (all from BioWhittaker), and 10% fetal bovine serum (HyClone Laboratories, Inc.).

Proliferation Assay—Cell proliferation assays were performed as described previously (7). Briefly, cells were serum-starved, followed by addition of ribozymes complexed with 7.2 μg/ml LipofectAMINE (3.6 μM DOSPA)1. Following a 3–4 h uptake period, ribozyme-lipid complexes were washed out, and cells were stimulated with fetal bovine serum. The percentage of proliferating cells was measured by bromodeoxyuridine incorporation. Control wells were treated with lipid only and were washed and incubated at 37°C for 2 h. The resulting internally labeled transcripts were purified over a G50 spin column (Pharmacia Biotech Inc.).

RESULTS

Target Site Selection Strategy—Hammerhead ribozymes can recognize and cleave RNA sequences containing U followed by A,C, or U. This consensus sequence occurs very frequently; the murine c-myb transcript contains 152 instances of this sequence. We chose to test five sites within rat c-myb, other four sites were conserved. Active and inactive forms of the ribozymes targeting the five sites within rat c-myb were synthesized with and without phosphorothioate linkages in the binding arms. The inactive ribozymes contained two nucleotide changes in the catalytic core that eliminate cleavage activity. The effect of the ribozymes on proliferation of rat aortic smooth muscle cells was assessed (data not shown). Although four of the five active ribozymes showed a significant inhibition of proliferation, the degree of inhibition was relatively low (approximately 15–30% inhibition by the active ribozyme compared to the inactive ribozyme control). Poor performance in cell culture could be the result of rapid intracellular degradation. Beigelman et al. (8) have reported extensive modifications designed to enhance the nuclease resistance of hammerhead ribozymes while retaining catalytic activity. We decided to test such modifications, focusing on the ribozyme targeting site 575.

Effect of Backbone and 2'-Sugar Modifications on Cell Culture Efficacy—Fig. 2 shows the site 575 with and without a nuclease-sterilizable core and with and without phosphorothioate linkages in the binding arms. All of the modifications showed some efficacy by active ribozyme versus inactive control, suggesting that the inhibitory effect was mediated by ribozyme cleavage of c-myb RNA. For both the U4 2'-C-allyl and the U4, U7 2'-amino variants, the ribozymes containing both a "stabilized" core and phosphorothioate linkages gave the most enhanced inhibition of smooth muscle cell proliferation. The ini-

1 The abbreviation used is: DOSPA, 2,3-dioleoyloxy-N-[(2-sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanoininium trifluoroacetate.

2 T. Jarvis, unpublished experiments.
TABLE I

| Cleavage site | Percent cleaved | Relative cleavage |
|---------------|-----------------|-------------------|
|               | RNase H | Ribozyme | RNase H | Ribozyme |
| 549           | 39      | 87       | 60      | 93       |
| 551           | 49      | 57       | 75      | 60       |
| 575           | 33      | 94       | 51      | 100      |
| 634           | 38      | 68       | 58      | 73       |
| 738           | 58      | 78       | 89      | 83       |
| 839           | 65      | 27       | 100     | 29       |
| 936           | 50      | 62       | 77      | 66       |
| 1017          | 64      | 40       | 98      | 43       |
| 1082          | 33      | 55       | 51      | 59       |
| 1203          | 11      | ND       | 17      | ND       |
| 1224          | 24      | ND       | 37      | ND       |
| 1334          | ND      | 6        | ND      | 9        |
| 1346          | 12      | ND       | 18      | ND       |
| 1389          | 7       | ND       | 11      | ND       |
| 1404          | 16      | ND       | 25      | ND       |
| 1553          | 52      | 87       | 80      | 93       |
| 1597          | 55      | 71       | 85      | 76       |
| 1598          | 52      | 80       | 80      | 85       |
| 1635          | 59      | 84       | 91      | 90       |
| 1721          | 50      | 62       | 77      | 66       |
| 1724          | 27      | 66       | 42      | 70       |
| 1811          | 14      | ND       | 22      | ND       |
| 1892          | 13      | ND       | 20      | ND       |
| 1895          | 26      | 40       | 40      | 42       |
| 1909          | 12      | 31       | 18      | 33       |
| 1943          | 15      | 66       | 23      | 70       |

Data are expressed as percent of the full-length transcript cleaved during a 1-h assay.

Results in Fig. 2 indicated that both a nuclease-resistant core and phosphorothioate linkages in the binding arms were advantageous for obtaining maximum cell culture efficacy. Since phosphorothioate linkages are associated with some degree of cytotoxicity and nonspecific effects (22, 23), we wished to determine the minimum number of phosphorothioates sufficient for cell efficacy. Fig. 3A shows a comparison of ribozymes containing 5 phosphorothioate linkages at the 5'-end, 5 phosphorothioate linkages at the 3'-end, or 5 phosphorothioate linkages at both the 5'- and 3'-ends. The ribozyme containing phosphorothioates only at the 3'-end showed almost no efficacy, while the ribozyme containing phosphorothioates at the 5'-end showed equivalent efficacy to that containing phosphorothioates at both the 5'- and 3'-ends. In this experiment, the inactive ribozyme showed some inhibition relative to the vehicle-treated control. A ribozyme with scrambled sequence binding arms exhibited an equivalent degree of inhibition, indicating that this effect was not mediated by ribozyme binding but was truly a "nonspecific" effect on proliferation. Next, we compared ribozymes with varying numbers of phosphorothioates at the 5'-end (Fig. 3, B and C). The degree of efficacy gradually decreased as the number of phosphorothioate linkages was reduced. From these experiments, we concluded that four to five phosphorothioate linkages at the 5'-end gives optimal efficacy in this cell culture system.

The ribozymes used in this study contained either 3'-phosphorothioate linkages or a 3'-5' inverted thymidine modification (24) to protect against 3'-exonuclease activity (8). We have subsequently shown that the outcome of this assay is not particularly sensitive to the presence or absence of this 3'-protect-
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FIG. 2. Cell efficacy of stabilized ribozymes. Ribozymes targeting c-myc site 575 were complexed with LipofectAMINE and delivered to rat aortic smooth muscle cells at a 100 nM dose. Cell proliferation was measured as described under "Materials and Methods." Active and inactive versions of several different chemical modifications were tested. 2'-O-Me indicates an RNA core with five 2'-O-methyl residues at the 5'- and 3'-ends at three different positions within the stem. U4,C-allyl indicates a core with a 5'- and 3'-end of U4 containing a C-allyl protecting group. Anti-c-myc ribozymes containing various protecting groups including a 3'-3' inverted thymidine, a 3'-3' inverted abasic residue, a 3'-butanediol or no 3' protecting group at all showed equivalent efficacy in inhibiting smooth muscle cell proliferation (data not shown). This may indicate that over the timecourse of this assay, the additional stability conferred by these modifications is not significant.

Optimization of Binding Arm Length—Ribozymes targeting c-myc site 575 were synthesized with arm lengths ranging from 5 to 12 nucleotides. The effects of these ribozymes on cell proliferation are shown in Fig. 4. The data are presented as specific inhibition by active versus inactive ribozyme. The optimal arm length was 6 to 7 nucleotides. We confirmed in five separate experiments that there was no significant difference in efficacy between the 6/6 (StemI/ StemIII) and 7/7 arm ribozymes (data not shown). We also tested ribozymes with asymmetric arm lengths (StemI/ StemIII with 5/10 or 10/5 nucleotides). Both asymmetric variants performed similarly to the 7/7 symmetric ribozyme (data not shown). The symmetric ribozyme containing seven nucleotide binding arms (no. 2972) was used as a standard for comparison in the experiment that follows. We have shown previously that this ribozyme inhibits rat, pig, and human vascular smooth muscle cells in a dose-dependent fashion and that the inhibition of proliferation correlates with a reduction in c-myc RNA levels (7).

Effect of 2'-Sugar and Base Modifications—Beigelman et al. (8, 16, 17) have developed a broad spectrum of different modifications in the catalytic core of hammerhead ribozymes that enhance resistance to nuclease degradation while preserving significant catalytic activity. Differences in intracellular stability, cleavage rate, uptake, and localization properties conferred by these modifications could alter the cell culture efficacy of the ribozyme. We tested several of these modifications, as shown in Fig. 5. A number of modifications showed equivalent cell efficacy to that exhibited by the U4 2'-C-allyl modified ribozyme. Others, such as U7 2'-C-allyl and U4 2'-fluoro, showed a somewhat lower magnitude of inhibition at the 100 nM dose. Experiments performed at lower doses supported the conclusion that none of the variants differed by more than 2-fold in the dose required to achieve 50% inhibition (data not shown). The inactive ribozyme containing the U4,U7 2'-amino modification
showed greater inhibition than inactive ribozymes containing any of the other modifications. This was seen with the related ribozyme in Fig. 2 as well. We believe that this inhibition represents a truly nonspecific effect because controls using a ribozyme containing the U4, U7 2'-amino modified chemistry and scrambled binding arm sequences showed similar levels of inhibition in side-by-side comparisons (data not shown).

The results in Fig. 5 indicate that none of the modified chemistries results in an improvement in cell efficacy in the smooth muscle cell proliferation assay compared to the U4 2'-C-allyl modification.

**Discussion**

We have used a systematic method to identify c-myb ribozymes that inhibit cell proliferation when delivered exogenously to cultured vascular smooth muscle cells. The inhibition is mediated by ribozymes containing a catalytically active core, while inactive controls fail to inhibit. The degree of inhibition observed can be affected profoundly by both backbone and 2'-sugar modifications. The optimal ribozyme configuration for inhibition of cell proliferation in this system consists of four phosphorothioate linkages at the 5'-end, six or seven nucleotide binding arms, 30 2'-O-methyl residues, and any of a variety of 2'-sugar or base modifications at positions U4 and U7.

The results in Figs. 2 and 3 demonstrate that both a "stabilized" core and several 5'-phosphorothioate linkages are advantageous for achieving substantial ribozyme-mediated inhibition of smooth muscle cell proliferation. Beigelman et al. (8) have shown that the U4-C-allyl and the U4, U7-amino ribozyme motifs increase the serum half-life of the ribozyme >16,000-fold relative to an all RNA ribozyme. Addition of 5'-phosphorothioate linkages increase the resistance to 5'-exonuclease activity. Thus, the enhanced cell efficacy observed with the combination of these two types of modifications could be attributed solely to enhanced resistance to nuclease. However, we cannot rule out the possibility that the phosphorothioate moieties are advantageous for some additional reason, such as conferring altered intracellular localization or trafficking properties.

The optimal hammerhead ribozyme binding arm length for intracellular activity may be a function of the thermodynamic stability of the duplex formed upon substrate binding, the kinetics of substrate binding and release, or of competing intramolecular ribozyme structures that can compromise substrate binding or conformation of the catalytic core. We have found that there is a distinct arm length optimum of six or seven nucleotides in each arm for the ribozyme targeting c-myb site 575. Depending on the sequence of the binding arms, the optimal arm length could vary for ribozymes targeting different sites.

We have shown that a variety of different chemical modifica-

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3 Although the majority of experiments performed with this ribozyme chemistry showed greater inhibition by the active version compared to the inactive, we have occasionally observed that inhibition by the inactive ribozyme is equal to that of the active, especially at higher doses (K. G. Draper, L. Beigelman, J. Matulic-Adamic, J. McSwiggen, V. Thackray, D. T. Stinchcomb, T. C. Jarvis, and N. Usman, manuscript in preparation).

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4 K. G. Draper, L. Beigelman, J. Matulic-Adamic, J. McSwiggen, V. Thackray, D. T. Stinchcomb, T. C. Jarvis, and N. Usman, manuscript in preparation.

5 LipofectAMINE delivery results in virtually 100% of the cells taking up ribozyme, with a fairly homogeneous distribution within the population, as demonstrated by flow cytometry using a fluorescently labeled ribozyme (26). In addition, uptake studies using radioactive ribozymes show that the sheer number of ribozymes delivered to each cell exceeds the c-myb mRNA copy number by many orders of magnitude (data not shown). Therefore, a very small percentage of the ribozyme that is taken up could represent the "bioactive" fraction responsible for the observed efficacy. Although ribozyme intracellular localization can be studied using confocal microscopy, or by careful fractionation, it is difficult to establish a meaningful correlation between cell efficacy and the observed localization of the bulk population.
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potentially enhance ribozyme delivery, efficacy, and residence time in cells and tissues.

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