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Cleavage of human *MDR*1 mRNA by a hammerhead ribozyme

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We designed a hammerhead ribozyme which site-specifically cleaved the GUC sequence in codon 179 of *MDR*1 mRNA. The cleavage site was 6 amino acids upstream from the drug binding site and was considered sufficiently close to the essential locus for *P*-glycoprotein function. The ribozyme cleaved the *MDR*1 mRNA under physiological conditions in vitro. The cleavage was dependent on ribozyme concentration and on incubation time. Mg^{2+} ion was essential for the cleavage. These results show that a potentially useful tool is at hand which may inactivate *MDR*1 mRNA and revert the multidrug resistance phenotype.

Hammerhead ribozyme; Multidrug resistance

1. INTRODUCTION

When tumor cells are exposed to one of many heterocyclic cytotoxic drugs the cells become resistant to other structurally and functionally unrelated drugs [1–3]. This phenomenon, called MDR, is one of the major obstacles in cancer chemotherapy. The underlying mechanism of MDR is the cellular overproduction of *P*-glycoprotein, a 170 kDa transmembrane protein that serves as an energy-dependent drug efflux pump [4]. By rapid elimination of drugs that enter the cell, tumor resistance can occur despite high blood levels of drugs. *P*-Glycoprotein is encoded by a small group of closely related genes termed *MDR*; only *MDR*1 is known to confer drug resistance [5–9]. Theoretically, *P*-glycoprotein activity can be blocked at three levels: DNA, RNA and protein. Since amplification of the *MDR*1 gene is not a prerequisite to *P*-glycoprotein-related resistance in human tumor cells [10,11], the potential loci of inhibition can be narrowed to the mRNA and protein levels.

One means to intercept a target mRNA is to use a catalytic RNA, or ribozyme [12,13]. Since ribozymes recognize a GUC sequence and cleave it most efficiently, attempts were made to determine whether a GUC sequence is located within any critical region on *MDR*1 mRNA. We observed that one GUC sequence is located at codon 179, 6 amino acids upstream from the drug binding site at amino acid codon 185. On the premise that the GUC locus was sufficiently close and important to the drug binding site, we designed a hammerhead ribozyme of *MDR*1 mRNA for the GUC sequence in codon 179. The ribozyme that we designed cleaved *MDR*1 RNA, in *trans*, at the specific site in vitro.

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Abbreviations: MDR, multidrug resistance; FBS, fetal bovine serum; VCR, vincristine; TMQ, trimetrexate; RT-PCR, reverse transcription polymerase chain reaction; CTAB, cetyl-trimethylammonium bromide.

2. MATERIALS AND METHODS

2.1. *Cells and RNA extraction*

The parent MOLT-3 human acute lymphoblastic leukemia cell line [14] and drug-resistant sublines were maintained in RPMI-1640 medium containing 10% (v/v) FBS and were fed twice a week with fresh medium. The multidrug-resistant sublines used were MOLT-3/$VCR_{1,000}$ (1,000-fold VCR resistant) [15] and MOLT-3/TMQ_{800} (800-fold TMQ resistant) [16]. Total cellular RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform method [17].

2.2. *RT-PCR*

For the RT-PCR of *MDR*1 transcripts, cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase (Gibco, Grand Island, NY) with random hexamer (Pharmacia, Piscataway, NJ) as a primer. Exons 6 through 8 were then amplified from this cDNA by using GeneAmp PCR Core Reagent Kit (Perkin-Elmer Cetus, Norwalk, CT) with oligonucleotide primers from exon 6 (5′-TTCATGCTATAATGCGACAGGAGATA) and exon 8 (5′-TTCTTTATCAGTAAATGAAGATAGTA). The clear and distinct 266-bp product was identified in 1.8% agarose gel and 1 × Tris-acetate EDTA buffer.

2.3. *Cloning of PCR products and sequencing*

The PCR products were ligated directly to pT7Blue T-vector (Novagen, Madison, WI). After transformation of *E. coli*, colonies were selected and screened. Plasmid DNA was prepared by CTAB-DNA precipitation method and double-stranded DNA sequencing was performed directly after alkali denaturation by using Sequenase version 2.0 kit (U.S. Biochemical, Cleveland, OH) [18]. At least 6 clones of each subline were sequenced and analyzed for the orientation. The

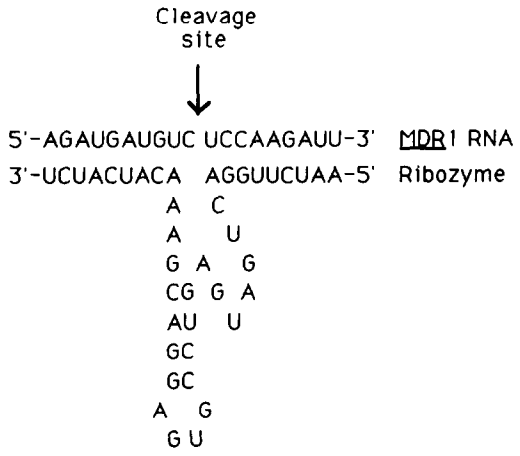

Fig. 1. Structure of the *MDR*1 ribozyme and the complementary *MDR*1 RNA with the GUC cleavage site.

clones that could produce the sense transcript were chosen for making *MDR*1 RNA substrate as described below.

2.4. *In vitro transcription of RNA (*MDR*1 or ribozyme) from plasmid templates or synthetic DNA template*

Transcriptions of RNAs from plasmid templates that contained T7 RNA polymerase promoter were carried out by using Riboprobe Gemini II Core System (Promega, Madison, WI). The transcription reaction mixture contained 5 mg linearized plasmid DNA cut with *Bam*HI, 0.5 U/μl T7 RNA polymerase, 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 10 mM dithiothreitol, 0.5 mM ATP, GTP and UTP, 0.1 mM CTP, 100 mCi of [α-^{32}P]CTP (specific activity 800 Ci/mmol) (DuPont, Boston, MA); and 1 U/ml recombinant ribonuclease inhibitor in a 100 μl volume. The reactions were carried out at 30°C for 3 h. The products were treated with RQ1 RNAse-free DNAse (Promega) followed by phenol-chloroform extraction and ammonium acetate ethanol precipitation.

To synthesize the *MDR*1 ribozyme a set of primers was used. One primer contained the bacteriophage T7 RNA polymerase promoter sequence (5'-ATTAATACGACTCACTATAGAATCTTGGACTG-ATGAG) and the other primer was 5'-AGATGATGTTTCGTCCT-CACGGACTCATCAGTC. The primers were mixed to form a heteroduplex, and PCR amplification was performed. The transcription of RNA from the synthetic DNA template was carried out under similar conditions except we used the same amount of cold rNTP's without [α-^{32}P]CTP. The synthetic DNA template concentration in the reaction mixture was 0.02 mg/ml.

2.5. *In vitro cleavage reactions*

The ribozyme and substrate RNA were mixed in a 10 μl reaction volume containing 50 mM Tris-HCl, pH 7.5, and 1 mM EDTA. The mixture was heated to 95°C for 2 min, quick-cooled on ice, various concentrations of $MgCl_2$ were added, and then it was incubated at 37°C for various amounts of time. The reactions were stopped by the addition of an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol blue, 0.05% xylene cyanol), heated at 65°C for 5 min, analyzed in a 6% polyacrylamide-7 M urea gel in Tris-borate EDTA buffer and autoradiographed.

3. RESULTS AND DISCUSSSION

In the present study, a hammerhead ribozyme was designed to cleave the GUC sequence of the codon 179 in exon 7 of the *MDR*1 gene in MOLT-3 human acute lymphoblastic leukemia cells resistant to TMQ or VCR (Fig. 1). This site is only 6 amino acids away from the drug binding site that is considered an essential locus for *P*-glycoprotein function. We reasoned that, if we specifically cleaved the mRNA at a crucial site, the resulting mRNA fragments would be non-functional, even if the fragments happened to be translated into polypeptides. The hammerhead ribozyme we designed was based on the *trans*-acting ribozyme described by Haseloff and Gerlach [13]. It had the same ribozyme's core sequence as theirs and flanking sequences totalling 18 nucleotides, 9 on both ends of the catalytic center. These 18 nucleotides formed base pairs specifically with the *MDR*1 substrate.

We have previously shown that both MOLT-3/TMQ_{800} and MOLT-3/$VCR_{1,000}$ display MDR1 mRNA overexpression and *P*-glycoprotein overproduction [15]. In this study we confirmed this in that the 266-bp PCR products of *MDR*1 could be amplified only from the resistant cells but not from the parent cell line (data not shown). The primers used are specific only for *MDR*1, not for *MDR*2, which does not confer MDR phenotype. Furthermore, sequencing the RT-PCR products of MOLT-3/TMQ_{800} and MOLT-3/$VCR_{1,000}$ showed that there were no mutations between codon 156 and 243 of *MDR*1 (Fig. 2), indicating that the ribozyme designed could work efficiently.

Transcription from the plasmid template that contained the *MDR*1 RT-PCR product using T7 RNA polymerase and [α-^{32}P]CTP generated a radioactive 329-base *MDR*1 RNA substrate. This substrate and the

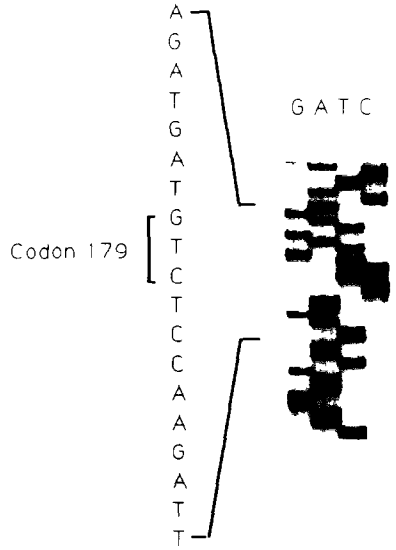

Fig. 2. cDNA sequence analysis of the *MDR*1 gene of MOLT-3/TMQ_{800} cells in the target region of the *MDR*1 ribozyme. The sequencing template used was double-stranded pT7Blue T-vector plasmid DNA containing the RT-PCR product of the *MDR*1 mRNA (codon 156–243). Dideoxy sequencing was performed.

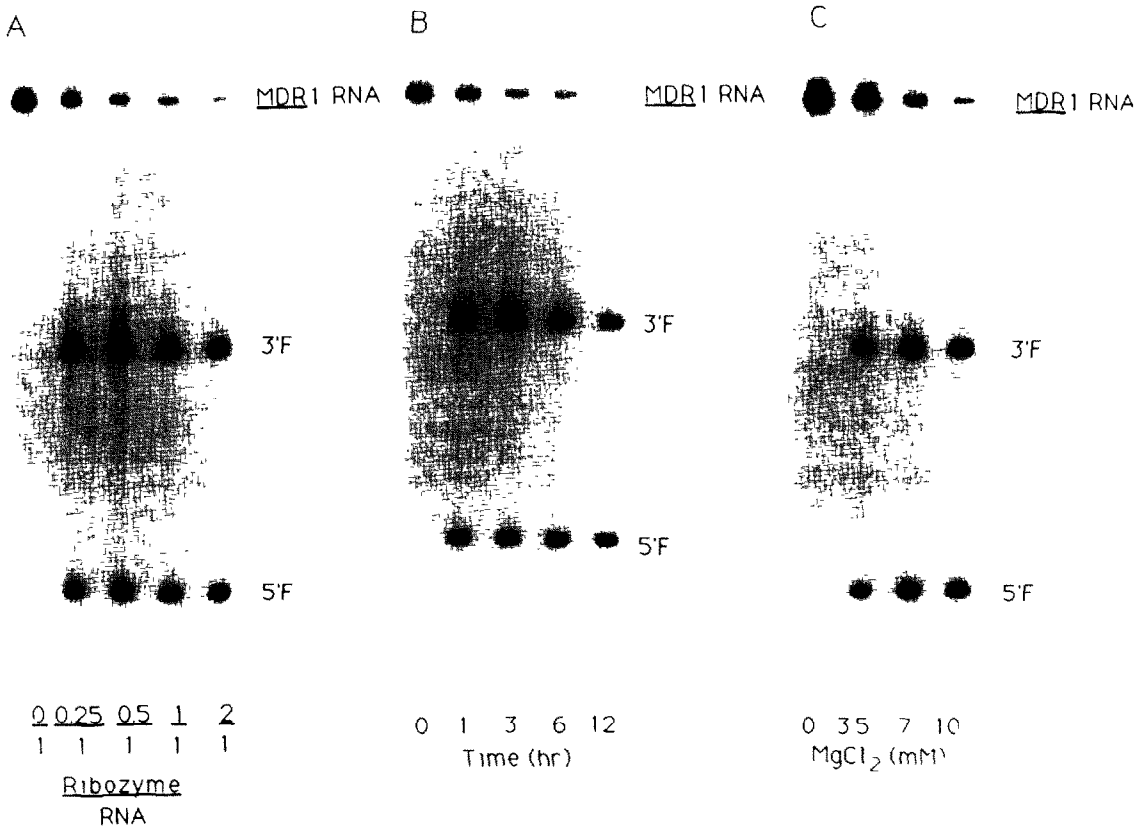

Fig. 3. Cleavage of *MDR*1 substrate (329 bases) by the *MDR*1 ribozyme to two cleavage products. 5′ fragment (5′F) is 128 bases long and contains the 5′ portion of the *MDR*1 substrate and plasmid sequence. 3′ fragment (′F) is 201 bases long and contains the 3′ portion of the *MDR*1 substrate. Only the substrate was labeled with [α-^{32}P]CTP. (A) Cleavage reaction with increasing proportions of the ribozyme. Ribozyme/RNA ratio is shown on a molar basis. The reaction was run for 12 h in physiological pH and temperature in the presence of 10 mM $MgCl_2$. (B) Reaction time vs. activity profiles of the ribozyme. Equimolar amounts of ribozyme and substrate were incubated in the presence of 10 mM $MgCl_2$. (C) Optimization of $MgCl_2$ concentration for the cleavage reaction. Equimolar amounts of ribozyme and substrate were incubated with various concentrations of $MgCl_2$ for 12 h.

ribozyme were used to determine the specificity of ribozyme cleavage and to optimize cleavage conditions in vitro. Incubation of the *MDR*1 RNA substrate with the MDR ribozyme at physiological pH and temperature resulted in specific cleavage of the substrate to 201 and 128 base products (Fig. 3A). Increases in the amounts of the ribozyme relative to substrate resulted in progressively large amounts of cleavage products. The cleavage reaction was also time dependent. Thus, substantial cleavage occurred within 1 h and progressed continually over 12 h of incubation period (Fig. 3B). The cleavage reaction also had an absolute requirement for Mg^{2+} (Fig. 3C).

Chang et al. designed the same type of hammerhead ribozyme against HIV-1 gag gene which had 16 base flanking sequences [19]. Under the same conditions we used, they observed substantial cleavage of the 138-nucleotide long gag substrate after 30 min and complete cleavage in 14 h. The cleavage reaction we observed may be somewhat slower than theirs. This may be due to 2 extra nucleotides in the flanking sequences of our ribozyme as compared to theirs. Goodchild and Kohli [20] examined the effects of the length of base-paired flanking sequences on ribozyme kinetics. They observed that reduction of the length of flanking sequences from 20 to 12 base pairs resulted in 10-fold increases in the rate of cleavage reaction.

Ribozymes have been proposed as anti-HIV agents [21] as well as agents to cleave mutated ras oncogene [22]. The availability of a ribozyme active against *MDR*1 mRNA offers hope that the MDR phenotype may be reverted by this agent. We have begun work towards this goal.

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