Engineered RNase P ribozymes increase their cleavage activities and efficacies in inhibiting viral gene expression in cells by enhancing the rate of cleavage and binding of the target mRNA

Hua Zou, Jarone Lee, Ahmed F. Kilani, Kihoon Kim, Phong Trang, Joseph Kim, Fenyong Liu

Program in Infectious Diseases and Immunity
Program in Comparative Biochemistry
School of Public Health
140 Warren Hall
University of California
Berkeley, CA 94720

Correspondence should be addressed to:
Dr. Fenyong Liu
School of Public Health
140 Warren Hall
University of California
Berkeley, CA 94720
Tel: (510)-643-2436
Fax: (510)-643-9955
E mail: liu_fy@uclink4.berkeley.edu

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Abbreviations: ptRNA, precursor tRNA.
Summary

Engineered RNase P ribozymes are promising gene-targeting agents that can be used in both basic research and clinical applications. We have previously selected ribozyme variants for their activity in cleaving an mRNA substrate from a pool of ribozymes containing randomized sequences. In this study, one of the variants was used to target the mRNA encoding thymidine kinase (TK) of herpes simplex virus 1 (HSV-1). The variant exhibited enhanced cleavage and substrate binding, and was at least 30 times more efficient in cleaving TK mRNA \textit{in vitro} than the ribozyme derived from the wild type sequence. Our results provide the first direct evidence to suggest that a point mutation at nucleotide 95 of RNase P catalytic RNA from \textit{Escherichia coli} (G95 -> U95) increases the rate of cleavage while another mutation at nucleotide 200 (A200 -> C200) enhances substrate binding of the ribozyme. A reduction of about 99% in TK expression was observed in cells expressing the variant while a 70% reduction was found in cells expressing the ribozyme derived from the wild type sequence. Thus, RNase P ribozyme variant is highly effective in inhibiting HSV-1 gene expression. Our study demonstrates that ribozyme variants increase their cleavage activity and efficacy in blocking gene expression in cells through enhanced substrate binding and rate of cleavage. These results also provide insights into the mechanism of how RNase P ribozymes efficiently cleave an mRNA substrate and furthermore, facilitate the development of highly active
RNase P ribozymes for gene-targeting applications.
Introduction

Nucleic acid-based gene interference strategies, such as antisense oligonucleotides, ribozymes or DNAzymes, and RNA interference (RNAi), represent powerful research tools and promising therapeutic agents for human diseases (1-8). Each of these approaches has its own advantages and limitations in term of targeting efficacy, sequence specificity, toxicity, and delivery efficiency in vivo. Further studies, such as constructing new and more effective ribozymes, would significantly facilitate the development of these approaches for gene-targeting applications.

RNase P is a ribonucleoprotein complex responsible for processing transfer RNA (tRNA) by catalyzing a hydrolysis reaction to remove a 5´ leader sequence from the precursor tRNA (ptRNA) to form a mature tRNA molecule (9,10). In *Escherichia coli*, RNase P consists of a catalytic RNA subunit (M1 RNA) and a protein subunit (C5 protein) (11). Previous studies have revealed that the structures of the substrates, rather than their specific sequences or nucleotides, dictate the recognition and cleavage by the enzyme (12). These findings suggest that, for any given sequence that satisfies the structural requirements, M1 RNA can be used to target the sequence for a specific and irreversible cleavage. Indeed, Altman and colleagues proposed that M1 RNA can be used to cleave an mRNA sequence if a custom-designed external guide sequence (EGS) is constructed to hybridize to the target mRNA and guide M1 RNA for the specific cleavage (Figure 1A) (13). Furthermore, M1 RNA can be covalently linked to a guide
sequence (GS) that is complementary to a target mRNA sequence (14,15). The resulting ribozyme, M1GS RNA, can bind the mRNA substrate through Watson-Crick base pairing interaction between the guide sequence and the mRNA (Figure 1A). Thus, the intrinsic catalytic activity of M1GS, in principle, can be used to target virtually any known mRNA sequence. In this reaction, the ribozyme uses its guide sequence to bind to its mRNA substrate through base pairing interactions and then docks the substrate into the active site, followed by cleavage and finally by product dissociation. We have previously shown that M1GS ribozymes cleaved the mRNA sequences of herpes simplex virus 1 (HSV-1) and human cytomegalovirus (16-18) and inhibited viral gene expression in cultured cells (19-21). For example, a M1GS ribozyme that cleaved the mRNA sequence (TK mRNA) that encodes HSV-1 thymidine kinase (TK) was constructed (15,18). A reduction of 70-75% in the expression levels of TK mRNA and protein was observed in cells that expressed the ribozyme.

Targeted cleavage of mRNA by RNase P ribozyme provides a unique approach to inactivate any RNA of known sequence expressed in vivo. In order to develop this ribozyme for practical use both as a research tool and as a therapeutic agent for gene-targeting applications, further studies, such as genetic engineering of the ribozymes for efficient enzymatic activity and investigating how ribozymes interact with mRNA substrates and achieve efficient cleavage, are needed to improve M1GS RNA catalytic efficiency in vitro and its efficacy in vivo. Using a nuclease footprint analysis and a
site-specific UV crosslinking approach, we have previously mapped the regions of the ribozymes that are in close contact with an mRNA model substrate (16-18). Moreover, using an in vitro selection procedure, we have recently isolated M1GS ribozyme variants that are more efficient in cleaving a specific mRNA sequence than that derived from the wild type M1 RNA (19). Little is currently known about how these variants increase their activity in cleaving the target mRNA substrate. Further characterization of these variants will provide insight into the mechanism of how RNase P ribozymes cleave an mRNA substrate efficiently and generate general guidelines for construction of highly active ribozymes for gene targeting applications.

In the present study, we used one of these ribozyme variants to target the HSV-1 TK mRNA sequence and investigated its activity in cleaving the target mRNA in vitro and its efficacy in inhibiting TK gene expression in cultured cells that were infected with HSV-1. Our results indicate that the variant is at least 30 fold more efficient in vitro than the ribozyme derived from the wild type M1 sequence. More importantly, a reduction of about 99 % in TK mRNA and protein expression was observed in cells that expressed the variant. Biochemical characterization of the variant suggests that a point mutation at nucleotide position 95 of M1 RNA (G95 -> U95) may increase the rate of cleavage and another mutation at nucleotide position 200 (A200 -> C200) enhances substrate binding of the ribozyme. Our results provide the direct evidence that RNase
P ribozyme variant is highly effective in inhibiting HSV-1 gene expression and furthermore, demonstrate the utility of M1GS RNAs as a new class of gene-targeting ribozymes for both in vitro and in vivo applications. Generating highly active ribozymes and understanding the mechanism of how these ribozymes achieve their cleavage activity \textit{in vitro} and in cell culture will provide insight into developing RNase P ribozymes for gene-targeting applications.

\textbf{Experimental Procedures}

\textbf{Antibodies, viruses, and cells.} The monoclonal antibodies against human actin and HSV-1 ICP27 were purchased from Sigma, Inc. (St Louis, MO) and the Goodwin Institute of Cancer Research, Inc. (Plantation, Florida), respectively. The polyclonal antibody against HSV-1 TK was kindly provided by Dr. William Summers (Yale University). Vero, PA317, and ψCRE cells were maintained in Dulbeccos modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY) as described previously (15). The propagation of HSV-1 (strain F) was carried out in Vero cells, as described previously (15).

\textbf{Generation of ribozymes.} Plasmid pFL117, pV41, and pC102, which contain the DNA sequences encoding M1 RNA, variant V41, and mutant C102, respectively, have been described previously (18,22). Variant constructs pM95 and pM200 were generated by changing G95 -> U95 and A200 -> C200 in the M1 sequence of pFL117, respectively.
C102 contains several point mutations (e.g. A347C348 -> C347U348, C353C354C355G356 -> G353G354A355U356) at the catalytic domain (P4 helix) of the M1 RNA sequence (22).

The DNA sequences that encode ribozymes M1-TK, V41-TK, M95-TK, M200-TK, and C-M1-TK were constructed by PCR using pFL117, pV41, pM95, pM200, and pC102 as the templates, respectively, with OliT7 (5’-TAATACGACTCACTATAG-3’) as the 5’ primer and Oligo31 (5’-GTG GTGTCTGCGTT CGACTATGACCAG-3’) as the 3’ primer. Similarly, the DNA sequences that encode C-V41-TK, C-M95-TK, and C-M200-TK were generated by PCR using oligonucleotide AF25 (5’-GGAATTCTAATACGACTCACTATAG-3’) as the 5’ primer, Oligo31 as the 3’ primer, C-V41, C-M95, and C-M200 DNAs as the templates, respectively. The DNA sequences that encoded ribozyme C-V41, C-M95, and C-M200 were generated by PCR using oligonucleotide AF25 (5’-GGAATTCTAATACGACTCACTATAG-3’) as the 5’ primer, oligonucleotide C102 (5’-TATGACCATGATTACGCCAAGCTTCAGGTAAACTGACCGA
GAATCATCCTGCTAGCGTGGACAGTCA-3’) as the 3’ primer, pV41, pM95, and pM200 as the templates, respectively.

**Construction of RNA substrates.** Plasmid pFL120 contains the DNA sequences that encode the RNA substrate stk. The DNAs that code for stk3 were generated by PCR to replace the entire 13 nucleotide-long 3’ tail sequence of stk with a thymidine
nucleotide. The DNA sequences that code for RNA stk5 and stk3 were constructed by PCR using pFL120 as the template, AF25 as the 5’ primer, and oligonucleotides olistk5 (5’-CCGCGCAGCCTGGTCGAACGCAGACTATAGTGAGTC GTATTA-3’) and olistk3 (5’-AGTCGAACGCA-3’) as the 3’ primers, respectively.

Construction of retroviral plasmids and ribozyme-expressing cells. Retroviral constructs RvM1-TK, RvC-M1-TK, RvC-M200-TK, RvC-V41-TK, RvM200-TK, and RvV41-TK were constructed by placing the DNA sequences that code for M1-TK, C-M1-TK, C-M200-TK, C-V41-TK, M200-TK, and V41-TK under the control of the U6 promoter in the LXSN retroviral vector, respectively (15,23). Cells containing the M1GS-retroviral DNA sequence were generated as described previously (15,23). The retroviral vector DNAs that contained the ribozyme sequence were transfected into amphotropic PA317 cells using a mammalian transfection kit purchased from Gibco BRL (Grand Island, NY). At forty-eight hours post transfection, culture supernatants that contained retroviral vector particles were collected and used to infect ψCRE cells (15). At 48-72 hours postinfection, neomycin (Gibco BRL, Grand Island, NY) was added to the culture medium at a final concentration of 600 µg/ml. Cells were subsequently selected in the presence of neomycin for two weeks and neomycin-resistant cells were cloned.

The level of M1GS RNA expression in individual cell clone was determined by Northern analysis. Both nuclear and cytoplasmic RNA fractions from M1GS-expressing cells were isolated as described previously (21,24), and then separated in gels that
contained formaldehyde, transferred to nitrocellulose membranes, hybridized with the [\textsuperscript{32}P]-radiolabeled DNA probe that contained the DNA sequence coding for M1 RNA, and finally analyzed with a STORM840 phosphorimager. The radiolabeled DNA probe used to detect M1GS RNAs was synthesized from plasmid pFL117, by using a random primed labeling kit (Boehringer Manheim, Co., Indianapolis, IN). Only those cell clones that expressed similar levels of ribozymes were used for subsequent experiments.

**Viral infection and assays for TK expression.** Approximately \(10^6\) cells in a T25 flask were either mock-infected or infected with HSV-1 in 1.5 ml of Medium 199 at a multiplicity of infection (MOI) as specified in the Results. Cells were harvested at 8-16 hours postinfection. Total cellular RNA and protein samples were prepared from the cells as described previously (21,24). The RNA probes used to detect TK mRNA and the transcripts of the \(\alpha 47\), Us10, Us11 genes were synthesized from pTK129 and pTK141, and RNase protection assays were performed as described previously (15). The protected RNA products were separated in 8% urea/polyacrylamide denaturing gels, and quantitated with a STORM840 phosphorimager.

The denatured, solubilized polypeptides from cell lysates were separated on 9% [vol/vol] SDS-polyacrylamide gels cross-linked with \(N,N''\)-methylenebisacrylamide (25). The separated polypeptides were transferred electrically to nitrocellulose membranes and reacted with the antibodies against HSV-1 TK or ICP27. The
membranes were subsequently stained with a chemiluminescent substrate with the aid of a Western chemiluminescent substrate kit (Amersham Inc, Arlington Heights, IL) and quantitated with a STORM840 phosphorimagery. Quantitation was performed in the linear range of RNA and protein detection (e.g. two-fold changes in RNA and protein samples result in a two-fold change in signal bracketing the range of experimental values).

**Single-turnover kinetic analyses.** The cleavage reactions of substrate stk by different ribozymes were carried out in buffer A (50 mM Tris, pH 7.5; 100 mM NH4Cl, 100 mM MgCl2), and single-turnover kinetic analyses to determine the values of the observed cleavage rate ($k_{obs}$) were performed as described previously (19,26). Analyses were performed with a trace amount of radioactive substrate and an excess of ribozyme. Variations in the amount of substrate did not affect the observed cleavage rate ($k_{obs}$) at a fixed excess ribozyme concentration and the reaction followed pseudo-first-order kinetics. The concentrations of ribozyme tested ranged from 0.05-5 nM and the concentration of radioactive substrate was less than 0.01 nM. Pseudo-first-order rate constants of cleavage ($k_{obs}$) were assayed at each ribozyme concentration by the slope of a plot -ln[(Ft-Fe)/(1-Fe)] versus time using Kaleidagaph program (Synergy Software, Reading, PA). Ft and Fe represent the fraction of the substrate at time t and the end point (>10 hours) of the experiments, respectively. The values of the overall cleavage
rate \( (k_{\text{cat}}/K_m)^5 \) were calculated by the slope of a least-squares linear regression (kaleidigraph) of a plot of the values of \( k_{\text{obs}} \) versus the concentrations of the ribozymes (19,26).

**Determination of apparent reaction rate constant \( k_{\text{app}} \).** The experimental procedures to determine the apparent reaction rate constant \( k_{\text{app}} \) were carried out as described previously (19). Equimolar amounts of substrates and ribozymes were incubated in buffer B (16.6 mM PIPES, 40 mM Tris-HCl, pH 6.0, 100 mM NaCl, 100 mM CaCl2) at 37°C for 10 min to allow binding. At pH 6.0 and in the presence of CaCl2, the active ribozyme-substrate complexes can be formed but the cleavage rate is significantly reduced (16,19,27-30). After binding, the bound ribozyme-substrate complexes were separated from the unbound substrates using G-50 Sephadex gel filtration columns (Boehringer Mannheim, Indianapolis, IN). The eluted samples were examined in 5% polyacrylamide nondenaturing gels for the presence of labeled ribozyme-substrate complexes and the concentrations of the complexes were quantitated by a STORM840 phosphorimager. Ribozyme-substrate complexes were then diluted in different concentrations (2-70 nM) and incubated in buffer C (16.6 mM PIPES, 40 mM Tris-HCl, pH 6.0, 100 NaCl, 100 mM MgCl2) at 37°C. Aliquots were withdrawn from reaction mixtures at regular intervals (from 0 to 120 min). The cleavage products were separated on 15% denaturing gels, autoradiographed, and quantitated with a STORM840
phosphorimager. The apparent rate constant, \( k_{app} \), was calculated by the slope of the plot of \( \ln \left( \frac{S_0}{S_t} \right) \) versus time, where \( S_0 \) equals the initial substrate concentration and \( S_t \) equals the substrate concentration at a given time point. The values obtained were the average of three experiments.

**Binding assays and determination of binding dissociation constant (Kd).** The procedures to measure the equilibrium dissociation constants (Kd) of complexes of the ribozymes and the substrates were modified from Pyle et al (31). Various concentrations of M1GS RNAs were preincubated in buffer E (50 mM Tris, pH 7.5, 100 mM NH4Cl, 100 mM CaCl2, 3% glycerol, 0.1% xylene cyanol, 0.1% bromophenol blue) for 10 minutes before mixing with an equal volume of different concentrations of substrate RNA preheated under identical conditions. The samples were incubated for 10-120 minutes to allow binding, then loaded on a 5% polyacrylamide gel, and run at 10 Watts. The electrophoresis running buffer contained 100 mM Tris-Hepes pH 7.5 and 10 mM MgCl2 (31). The value of Kd was then extrapolated from a graph plotting percent of product bound versus M1GS RNA concentration. The values were the average of three experiments.

**Results**

**Efficient cleavage activity of the engineered RNase P ribozymes in vitro.** Since most mRNA species inside cells are associated with proteins and are present in a folded conformation, it is essential to choose a targeted region that is accessible to binding of
M1GS ribozymes. We have used an in vivo mapping approach with dimethyl sulphate (DMS) (15,32,33) to determine the accessible regions of TK mRNA. A position, 29 nucleotides downstream from the translation initiation site of TK gene, was chosen as the cleavage site for the M1GS ribozyme. The targeted region is one of the sequences most accessible to DMS modification, and is likely to be accessible to ribozyme binding (data not shown).

We have previously employed an in vitro selection procedure to isolate M1GS RNA variants that are more efficient in cleaving the TK mRNA sequence than the ribozyme derived from the wild type M1 RNA (19). The objective of the study was to generate highly active RNase P ribozyme variants that can be used to target an mRNA, and to study the variants to understand the catalytic mechanism of RNase P ribozymes in cleaving mRNA substrates. However, little is currently known about the mechanism of how some of these active variants increase their cleavage activity in vitro. We chose variant 41 (designated as V41) for the study because the ribozymes derived from this variant are among the most active M1GS RNAs in cleaving the TK as well as the mRNA sequence encoding chloramphenicol acetyltransferase (CAT) in vitro (see below, Table I).

Ribozyme V41-TK was constructed by covalently linking the 3’ terminus of V41 RNA to a guide sequence of 13 nucleotides that is complementary to the targeted mRNA sequence. V41 contains point mutations G95 -> U95 and A200 -> C200 (Figure 1C) (19).
Little is known about the functional role of G95 in M1GS RNA cleavage, while our recent UV crosslinking and nuclease footprint analyses suggest that A200 is in close proximity to the 3′ tail sequence of a mRNA substrate and is probably involved in binding the mRNA substrate (16-18). In order to study the function of these two mutations in the cleavage of the TK mRNA sequence, we constructed two additional ribozymes, M95-TK and M200-TK, which were derived from M1 RNA and contained the mutations G95 -> U95 and A200 -> C200, respectively. Two other M1GS ribozymes, M1-TK and C-M1-TK, were also constructed in a similar way and included in the study. M1-TK was derived from the wild type M1 while C-M1-TK was derived from C102 RNA, a M1 mutant that contains several point mutations at the P4 catalytic domain and is catalytically inactive (22). RNA substrate stk contains the targeted TK mRNA sequence of 46 nucleotides (Figure 1B). Cleavage of stk was observed in cleavage buffer A (50 mM Tris, pH 7.5; 100 mM NH4Cl, 100 mM MgCl2) in the presence of V41-TK, M95-TK, M200-TK, and M1-TK (Figure 2, lanes 2-3, Table I). In contrast, cleavage of the same substrate by C-M1-TK was barely detected due to the point mutations at the catalytic center region (Figure 2, lane 4). To determine whether the variant is more active in cleaving the TK mRNA sequence, cleavage of stk by the ribozymes was assayed and the overall cleavage rate $(k_{cat}/K_m)^S$ of the reactions was determined using kinetic analyses.
under single turnover conditions. Ribozyme V41-TK, M95-TK, and M200-TK were about 30, 5, and 8 times more active than M1-TK that was derived from the wild type M1 RNA sequence (Table I), indicating that ribozyme variants are much more efficient in cleaving TK mRNA than that derived from the wild type M1 RNA sequence.

**Increased rate of cleavage of the ribozyme variant.** The values of \((k_{\text{cat}}/K_m)^s\) obtained under single-turnover conditions may reflect the rates of substrate binding and cleavage of the substrate. Accordingly, experiments were carried out with the variant to determine whether a change in the rates of these steps contributed to the increased \((k_{\text{cat}}/K_m)^s\) values. To determine whether the mutations affect the rate of cleavage, substrate stk was allowed to form active complexes with the ribozymes in the presence of divalent ions and the apparent reaction rate constant, \(k_{\text{app}}\), for the complexes was measured. We mixed equimolar amounts of ribozymes and substrate stk in binding buffer B (16.6 mM PIPES, 40 mM Tris-HCl, pH 6.0, 100 mM NaCl, 100 mM CaCl2) in the presence of CaCl2 and under the buffer condition at pH 6.0, and separated the ribozyme-stk complexes from the unbound substrates using G-50 Sephadex columns. The buffer condition at pH 6.0 and in the presence of CaCl2 in buffer B, compared to that in regular cleavage buffer A (pH 7.5 and in the presence of MgCl2), was used in order to significantly reduce the rate of cleavage while allowing proper folding of the ribozymes and substrates and preserving the interactions between the ribozyme and the substrate in an active
ribozyme-substrate complex (14,16,19,27-30). For example, in the presence of CaCl2, the rate of cleavage of stk at pH 7.5 was at least 100 fold slower than that in the presence of MgCl2 (data not shown). Meanwhile, UV crosslinking and nuclease footprint analyses indicate that the regions of the ribozymes that potentially interact with the substrates in the presence of Mg++ ions are similar to those found in the presence of Ca++ ions, suggesting that the interactions between the ribozymes and substrates are similar in the presence of these different divalent ions (16,19,27-30).

To determine the values of \( k_{app} \), the ribozyme-substrate complexes were first diluted in different concentrations and then further incubated in the presence of 100 mM MgCl2 and under the buffer condition at pH 6.0 to allow cleavage. The apparent rate constant, \( k_{app} \), for M1GS-stk complexes was independent of the concentrations of the complexes within the tested range of 2-70 nM (Table II), suggesting that substrate cleavage takes place predominantly in the bound complexes (cis-cleavage) as observed previously (18,19). The values of \( k_{app} \) for the selected variants were at least 5x10^2 fold higher than those of C-M1-TK (Table II). Moreover, the values of \( k_{app} \) for M200-TK were similar to those of M1-TK, suggesting that this variant exhibits a similar rate of cleavage as M1-TK. In contrast, the values of \( k_{app} \) for V41-TK and M95-TK were about five times higher than those of M1-TK (Table II). M95-TK only contains the mutation G95 -> U95, which is also found in V41-TK. These results suggest that this mutation may
be responsible for the observed increased cleavage activity of M95-TK. This is further supported by our observation that a "rescued" mutant derived from M95-TK, in which U_{95} was changed back to G_{95}, exhibited similar activity as M1-TK (data not shown).

Enhanced substrate binding of the ribozyme variant. An increase in the overall cleavage rate \((k_{cat}/K_m)^8\) of the variants may also be due to additional tertiary interactions between the ribozyme and the substrate. These interactions may result in better binding and docking of the substrate to the active site of the ribozyme. Ribozyme-substrate complexes were separated in polyacrylamide gels under non-denaturing conditions and the binding affinities of the ribozymes to substrate stk, measured as the dissociation constant \((K_d)\), were determined and shown in Table I. M200-TK and V41-TK exhibited about 25 times higher binding affinity to stk than M1-TK. Since M200-TK only contains mutation \(A_{200} \rightarrow C_{200}\), which is also found in V41-TK, it is likely that this mutation is responsible for the observed increased affinity and activity of M200-TK. A ribozyme, M200r-TK, was derived from M200-TK, in which \(C_{200}\) was changed back to \(A_{200}\) and the wild type sequence was restored. M200r-TK exhibited identical affinity and activity as M1-TK (data not shown), suggesting that the mutated \(C_{200}\) functions to enhance substrate binding and is indeed responsible for the increased activity of M200-TK. In our single-turnover kinetic analysis, the overall rate
of the reactions \((k_{\text{cat}}/K_{\text{m}})^5\) is dictated by the rate of cleavage and the rate of the substrate binding (association) and may not be significantly affected by the rate of dissociation between the ribozyme and substrate. Our observations that M200-TK exhibits similar rate of cleavage as M1-TK (Table II) but shows better substrate binding suggest that the mutation \((A_{200} \rightarrow C_{200})\) accelerates the step of substrate binding (association), contributing to an increased overall cleavage efficiency.

**Importance of the 3’ tail sequence for increased substrate binding.** To further determine which part of the substrate is important for the increased binding affinity between the substrates and the ribozymes, we carried out the binding assays using substrates stk3 and stk5, which were derived from stk by deleting the 3’ tail and 5’ leader sequence, respectively (Figure 1B). The binding affinity of M1-TK to stk3 was about four times weaker than its affinity to stk (Table I). The binding affinities of M200-TK and V41-TK to stk3, however, were about 90 times weaker than those to stk. Indeed, the Kd values of M200-TK and V41-TK to stk3 were similar to those of M1-TK. In contrast, V41-TK and M200-TK still exhibited a 20 times higher binding affinity to stk5 than M1-TK (data not shown), suggesting that the 5’ leader sequence is not important for the increased binding affinity of the variants. These results strongly imply that the presence of the 3’ tail sequence contributes to the increased substrate binding and that deleting this sequence results in the loss of the increased affinity. This notion is further supported
by the results from kinetic analyses of the cleavage of stk3 by the variants. M200-TK did not exhibit higher activity \([k_{\text{cat}}/K_m]^{8}\) than M1-TK in cleaving stk3 while this mutant was about 8 times more active in cleaving stk than M1-TK (Table I). Cleavage of this substrate by M200-TK and V41-TK was about 15 times slower than their cleavage of stk, respectively. In contrast, the rate of cleavage of stk3 by M1-TK was only about two fold slower than that of stk. Thus, the presence of the 3’ tail sequence appears to be important for the increased cleavage efficiency of M200-TK and V41-TK. V41-TK still exhibited at least 20 fold higher cleavage activity than M1-TK when a 450 nucleotide long substrate of TK mRNA sequence was used (data not shown). This substrate includes the stk sequence and in addition, contains a 150 nucleotide long 5’ leader and a 287 nucleotide long 3’ tail sequence. These results suggest that V41-TK may also cleave the full-length TK mRNA more efficiently than M1-TK.

**Expression of the selected M1GS in tissue culture.** To express the M1GSs in cultured cells, the DNA sequences coding for the highly active V41-TK and M200-TK as well as M1-TK and C-M1-TK were subcloned and placed under the control of the small nuclear U6 RNA promoter, which has previously been shown to express M1GSs and other RNAs steadily (25,34-36). This promoter is transcribed by RNA polymerase III and its transcripts are highly expressed and primarily localized in the nucleus (37,38).
Two additional ribozymes, C-M200-TK and C-V41-TK were also cloned into the retroviral vector. C-M200-TK and C-V41-TK contain the mutations found in C102 and are catalytically inactive but contain the same guide sequence as M200-TK and V41-TK (Figure 2, lane 1). Indeed, C-M200-TK and C-V41-TK exhibited similar binding affinities to stk as M200-TK and V41-TK (Table I). Therefore, these ribozymes can be used as the control for the antisense effect of the guide sequence.

To construct cell lines that express M1GS ribozymes, amphotropic packaging cells (PA317) (23) were transfected with LXSN-M1GS DNAs to produce retrovirus particles that contained the genes for M1GS RNA. Subsequently, ecotropic packaging cells (ψCRE) (19,39) were infected with these retroviruses. Cells expressing the retroviruses were then selected in culture medium that contained neomycin and those that exhibited neomycin resistance were cloned. The level of M1GS RNA expression in individual cell clones was determined using Northern analysis with a DNA probe that is complementary to M1 RNA (Figure 3A). The expression of H1 RNA, the human nuclear RNase P RNA subunit, was used as the internal control (Figure 3B). The M1GS RNAs were exclusively expressed in the nuclei as they were only detected in the nuclear but not cytoplasmic RNA fractions (data not shown)(25). The constructed lines and a control line in which cells were transfected with LXSN vector DNA were indistinguishable in terms of cell growth and viability for up to two months (data not shown), suggesting that the expression of the ribozymes did not exhibit significant cytotoxicity. Only the cell
lines that expressed similar levels of these ribozymes were used for further studies in tissue culture.

**Increased efficacy of the ribozyme variants for inhibition of TK expression.** To determine the efficacy of the M1GSs for inhibiting TK expression, cells were infected with HSV-1 at a multiplicity of infection (MOI) of 0.5-1. Total RNAs were isolated from the cells that were mock-infected or infected with HSV-1 at 8 hours postinfection. Levels of TK mRNA were determined by an RNase protection assay. The levels of the overlapping transcripts coding for viral α47, Us10, and Us11 proteins, which expressions are not regulated by TK (40), were used as the internal controls for quantitation of TK mRNA expression. Figure 4 shows the results (which are summarized graphically in Figure 6) of the RNase protection experiments with both the TK and α47 probes. A reduction of about 99%, 92%, and 75% (average of three experiments) in the levels of TK mRNA expression was observed in cells that expressed V41-TK, M200-TK, and M1-TK, respectively (Figure 4, lanes 3 and 4). In contrast, cells that expressed C-M1-TK, C-M200-TK, and C-V41-TK only exhibited a reduction of less than 10% (Figure 4, lanes 1 and 2)(Figure 6). Thus, these observations suggest that the significant reduction of TK mRNA expression in cells that expressed M1-TK, M200-TK, and V41-TK was due to the catalytic cleavage of the target mRNA by these ribozymes. The low levels of inhibition found in cells that expressed C-M1-TK, C-
M200-TK, C-V41-TK RNAs were probably due to an antisense effect. This is because these control ribozymes contain the same guide sequence as M1-TK, M200-TK, and V41-TK but do not exhibit catalytic activity.

It is expected that the level of TK protein should decrease in M1GS-expressing cells because of the decreased level of TK mRNA. Protein extracts were isolated from cells either mock-infected or infected with HSV-1 at 16 hours postinfection. Viral proteins were separated electrophoretically in SDS-polyacrylamide gels and electrically transferred to two identical membranes. One of these membranes was stained with an anti-TK antibody (anti-TK) (Figure 5B) and the other was stained with a monoclonal antibody against viral ICP27 protein (anti-ICP27) (21) (Figure 5A). The latter is used to detect the expression of HSV-1 immediate-early protein ICP27, which serves as an internal control for the quantitation of TK protein expression. The expression of ICP27 is not regulated by the expression of TK protein (40). The results of three independent experiments are summarized in Figure 6: a reduction of about 99%, 91%, and 70% in the level of TK protein was observed in cells that expressed V41-TK, M200-TK, and M1-TK RNA, respectively. In contrast, a reduction of less than 10% was found in cells that expressed C-M1-TK, C-M200-TK, and C-V41-TK RNAs (Figure 6). The low level of reduction in the expression level of TK protein observed in cells that expressed these control ribozymes was presumably attributed to the antisense effect of the M1GS RNAs.

Discussion
The M1GS-based technology represents an attractive approach for gene inactivation since it generates catalytic and irreversible cleavage of the target RNA by using M1 RNA, a highly active RNA enzyme found in nature (9,10). M1 RNA may further increase its activity in cultured cells by interacting with the cellular proteins (9,15). These properties, as well as the simple design of the guide sequence, make M1GS an attractive and unique gene-targeting agent that can be generally used for antiviral as well as other in vivo applications.

Little is currently known about the rate-limiting step of M1GS RNA cleavage reaction in cultured cells. Equally unclear is how to increase M1GS RNA cleavage activity and efficacy in blocking gene expression in cultured cells. In this study, M1GS RNAs were targeted to an accessible region of TK mRNA and were expressed by the small U6 nuclear RNA promoter. This design would increase the probability for the ribozymes to locate and bind to their target mRNA sequence. Under the described settings, we hypothesized that the efficacy of RNase P ribozyme cleavage in cultured cells is dictated by its catalytic efficiency \([k_{\text{cat}}/K_m]\). If this is the case, increasing the cleavage activity of RNase P ribozymes may lead to a more effective inhibition of the target mRNA expression in vivo. Our results indicate that M200-TK and V41-TK, which are more efficient in cleaving TK mRNA \textit{in vitro}, are also more effective in inhibiting TK expression in cultured cells than M1-TK, the ribozyme derived from the wild type M1. Moreover, the ribozyme (V41-TK) that exhibited higher cleavage activities
[(k_{cat}/K_m)^s] appeared to be more effective in cell culture. These results strongly suggest that substrate binding and cleavage may represent the rate-limiting steps for M1GS ribozyme in cell culture. Moreover, our study implies that increasing the catalytic efficiency of RNase P ribozymes, by enhancing the substrate binding and/or the rate of cleavage, may lead to improved efficacy in inhibiting gene expression in cultured cells. The difference between the in vivo efficacies of V41-TK and M1-TK (e.g. 99% vs. 75%) appears to be more limited than that of the in vitro cleavage efficiencies (more than 30 fold difference). One of the possible explanations is that about 1-2% of the target mRNA may not be accessible to ribozyme binding, possibly due to its rapid transport to the cytoplasm. Another possibility is that the efficacy of the ribozymes in cells, in contrast to their activity in vitro, may be influenced by the intrinsic intracellular decay of the target RNA, as hairpin ribozymes have been shown to eliminate mRNAs effectively in cells only when cleavage is much faster than normal degradation (41,42). Further studies will be needed to address these issues.

Our results provide the first direct evidence to suggest that mutation G95 -> U95 may increase the rate of cleavage of a mRNA substrate. In our experiments to study the effect of the mutations on the rate of cleavage, substrate stk was allowed to form active complexes with the ribozymes in the presence of CaCl2 and under the buffer condition at pH 6.0. Pre-forming the active complexes allows the binding of the substrate to the ribozyme, proper folding of the ribozymes and substrate, and the docking of the substrate
to the active site. Buffer B (pH 6.0, 100 mM CaCl2), rather than regular cleavage buffer A (pH 7.5, 100 mM MgCl2), were used to reduce the rate of cleavage while preserving the interactions between the ribozyme and the substrate in an active ribozyme-substrate complex. Previous studies have shown that the rate of catalytic chemistry in the reaction of a ptRNA by M1 RNA is too fast under physiological pH conditions (pH 7.0-8.0) to be measured accurately (27-30). At pH 6.0, the rate of catalytic chemistry is significantly reduced, and may represent the rate-limiting step in the cleavage reaction of a ptRNA by M1 RNA and therefore, can be accurately assayed (27-30). Meanwhile, changes in the buffer pH condition and the substitution of MgCl2 with CaCl2 do not affect the binding, docking, and folding of the ribozymes and substrates nor the formation of the active ribozyme-substrate complexes (16,19,27-30). Thus, an increased value of $k_{\text{app}}$ may represent a faster rate of catalytic chemistry. It is possible that an increased rate of catalytic chemistry may result from either an accelerated physical breakage of the phosphodiester linkage or a faster conformational change that is potentially rate-limiting. Further detailed kinetic analyses should clarify these issues and elucidate the mechanism of how the mutation increases the overall rate of cleavage and affects the step of catalytic chemistry.

We show that mutation $A_{200} \rightarrow C_{200}$ is important for binding of the 3' tail sequence.

$A_{200}$ is not in the conserved ribozyme regions (9,10) and its position is not yet completely
defined in the current models of the three-dimensional structure of RNase P ribozymes (Figure 1C) (43,44). Several lines of evidence presented in our study strongly suggest that the presence of C200 enhances the binding of the ribozyme to the 3’ tail sequence of the mRNA substrate. First, M200-TK but not a rescued mutant was more active in cleaving stk than M1-TK. M200-TK also exhibited higher affinity to stk but not a higher apparent rate of cleavage (kapp) than M1-TK (Table I and II). Therefore, the mutated nucleotide appears to be responsible for the increased substrate binding and cleavage efficiency [(kcat/Km)s]. Second, the presence of the 3’ tail sequence contributes to the increased binding affinity and cleavage activity [(kcat/Km)s] and deleting this sequence results in the loss of the increased affinity and cleavage efficiency (Table I). Third, recent UV crosslinking and nuclease footprint analyses suggested that A200 is in close proximity to the 3’ tail region of an mRNA model substrate (16-18). A decrease of k_d may result from either an increase in the rate of substrate binding (association) or a reduction in the rate of substrate dissociation or both. It is conceivable that an additional interaction (or binding) between the ribozyme and the mRNA substrate may accelerate substrate binding as well as reduce the rate of substrate dissociation.

In our single-turnover kinetic analysis, the overall rate of the reactions [(kcat/Km)s] is dictated by the rate of cleavage and the rate of substrate binding (association) and may not be
significantly affected by the rate of substrate dissociation. Our observations that M200-TK exhibits similar rate of cleavage as M1-TK (Table II) but shows better substrate binding suggest that the mutation (A200 -> C200) probably accelerates substrate binding (association) by interacting with the 3´ tail sequence of the target mRNA.

By studying one of the variants (i.e. V41), our results provide the direct evidence that engineered RNase P ribozyme can increase its cleavage activity by enhancing the substrate binding and the rate of cleavage. The increased activity of V41-TK appears to be independent of the primary nucleotide sequence of the 3´ tail region. When the variant is used to target the mRNA sequence encoding chloramphenicol acetyltransferase (CAT) that is different from the TK mRNA sequence, the ribozyme also exhibited higher cleavage activity than that derived from wild type M1 RNA (data not shown). These results suggest that the ribozyme variants enhance the recognition of the substrate possibly by interacting with the structural features (e.g. the 2’ hydroxyl groups) of the 3´ tail sequence rather than the bases of the nucleotides. More importantly, these observations suggest that the catalytic domain of the selected variants can be generally used to construct highly active ribozymes to target any mRNA sequence. Our results also suggest that improving the in vitro catalytic efficiencies of the ribozymes should lead to increase their gene knock-down efficacies in tissue culture. Thus, our study provides a direction for the engineering of highly active and effective
RNase P ribozyme variants by carrying out selection procedures and manipulation of the M1 catalytic domain to interact more efficiently with the mRNA substrates. Further studies of the RNase P variants both in vitro and in cultured cells should provide insights into the mechanism of how an RNase P ribozyme efficiently cleaves an mRNA substrate and develop guidelines for the construction of highly effective ribozymes in gene-targeting applications.

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References


Table legends

Table I. The overall cleavage rate \([k_{\text{cat}}/K_m]^S]\) and binding affinity \((K_d)\) in reactions of substrates stk and stk3 with RNase P ribozyme variants and the ribozyme (M1-TK) derived from the wild type M1 RNA sequence. Binding assays were carried out in buffer E (50 mM Tris, pH 7.5, 100 mM NH4Cl, 100 mM CaCl2), using a protocol modified from Pyle et al (31). Single-turnover kinetic analyses to determine the values of \([k_{\text{cat}}/K_m]^S\) were carried out in buffer A (50 mM Tris, pH 7.5, 100 mM NH4Cl, 100 mM MgCl2) as described previously (19,22). The values shown are the average derived from triplicate experiments.

Table II. The apparent rate constants \((k_{\text{app}})\) of the ribozyme-substrate complexes at different concentrations. The ribozyme-stk complexes were separated from the unbound substrates by G-50 Sephadex gel filtration columns and diluted in different concentrations before the rate of cleavage was assayed. The cleavage products were separated in 15% polyacrylamide gels and quantitated with a STORM840 phosphorimager.
Legend to Figures

Figure 1. (A). Schematic representation of a ptRNA substrate, a precursor to 4.5S RNA, a small model substrate (EGS:mRNA) for ribonuclease P and M1 RNA from *E. coli* and a M1GS RNA construct to which a target mRNA has hybridized. The site of cleavage by RNase P or M1 RNA is marked with a filled arrow. The mRNA substrate contains three sequence elements: a 5’ leader sequence, a targeted sequence, and a 3’ tail sequence. (B). Schematic representation of the substrates used in the study. The targeted sequences that bind to the guide sequences of the ribozymes are boxed. The regions upstream and downstream from the targeted sequence represent the 5’ leader sequence and the 3’ tail sequence, respectively. (C). The mutated positions (G95 and A200) (circled) of ribozyme variant V41 at the secondary structure of the RNA subunit (M1 RNA) of RNase P from *E. coli* (45,46).

Figure 2. (A). Cleavage of substrate stk by the ribozymes. Substrate (20 nM) was incubated alone (lanes 5), with 10 nM of C-M1-TK (lane 4), 5 nM of M1-TK (lane 3), 0.5 nM of V41-TK (lane 2), or C-V41-TK (lane 1). Cleavage reactions were carried out for 30 min in buffer A (50 mM Tris, pH 7.5, 100 mM NH4Cl, 100 mM MgCl2) at 37°C.

Figure 3. The expression of M1GSs in cultured cells. Northern analyses were carried
out using nuclear RNA fractions isolated from cell lines that expressed V41-TK (lanes 1 and 5), M200-TK (lanes 2 and 6), M1-TK (lanes 3 and 7), and C-M1-TK (lanes 4 and 8). RNA samples (30 µg) were either separated on 1% agarose gels that contained formaldehyde, transferred to a nitrocellulose membrane, and hybridized to the [32P]-radiolabeled probes that contained the DNA sequence coding for M1 RNA (A) or H1 RNA, which is the RNA subunit of human RNase P (9)(B). The expression of H1 RNA (B) was used as the internal control.

Figure 4. Expression of TK mRNA, as detected by an RNase protection assay. RNase protection assays were performed as described previously (25). At 8 hours postinfection, total RNA (40 µg) was isolated either from parental ψCRE cells (P; lanes 5 and 6) or from cell lines expressing C-V41-TK (lane 1), C-M1-TK (lane 2), V41-TK (lane 3), and M1-TK (lane 4). The cells were either mock-infected (lane 6) or infected with HSV-1 (MOI=0.5) (lanes 1-5). The protected products corresponding to TK mRNA (TK mRNA) and the overlapping transcripts of α47, Us10, and Us11 mRNA (α47 mRNA) were about 90 and 180 nucleotides long, respectively. RNA probes were used in great excess of the detected RNA species.

Figure 5. Levels of expression of TK protein as determined by Western blot analysis with a
chemiluminescent substrate. At 16 hours postinfection, protein samples were isolated from parental \( \psi \)CRE cells (P; lanes 5-6 and 11-12) or from cell lines expressing V41-TK (lanes 1 and 7), M200-TK (lanes 2 and 8), M1-TK (lanes 3 and 9), and C-M1-TK (lanes 4 and 10). The cells were either mock-infected (lanes 6 and 12) or infected with HSV-1 (MOI=1) (lanes 1-5, 7-11). Protein samples (20 \( \mu \)g) were separated in two identical SDS-polyacrylamide gels and transferred electrically to two identical membranes. One membrane was allowed to react with a monoclonal antibody (Anti-ICP27) against HSV-1 immediate-early protein ICP27 (A) while the other was stained with the polyclonal antibody (Anti-TK) against HSV-1(F) TK protein (B). Both antibodies were used in great excess of the detected antigens.

Figure 6. Schematic representation of the expression levels of HSV-1 TK mRNA and protein in viral infected cells that did not express a ribozyme [P(\( \psi \)CRE)] or expressed ribozyme C-M1-TK (C-M1-TK), C-M200-TK (C-M200-TK), C-V41-TK (C-V41-TK), M1-TK (M1-TK), M200-TK (M200-TK), and V41-TK (V41-TK). The RNA and protein samples were isolated from cells at 8 and 16 hours postinfection, respectively. The values shown are the averages from three independent experiments. Solid bars: TK mRNA; open bars: TK protein.
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<th>Enzyme</th>
<th>Substrate</th>
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(A) M1GS RNA

(B) H1 RNA
Engineered RNase P ribozymes increase their cleavage activities and efficacies in inhibiting viral gene expression in cells by enhancing the rate of cleavage and binding of the target mRNA

Hua Zou, Jarone Lee, Ahmed F. Kilani, Kihoon Kim, Phong Trang, Joseph Kim and Fenyong Liu

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