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Directing RNase P-Mediated Cleavage of Target mRNAs by Engineered External Guide Sequences in Cultured Cells

Xiaohong Jiang, Naresh Sunkara, Sangwei Lu, and Fenyong Liu

Abstract

Ribonuclease P (RNase P) complexed with external guide sequence (termed as EGS) represents a novel nucleic acid-based gene interference approach to modulate gene expression. In previous studies, by using an in vitro selection procedure, we have successfully generated EGS variants that are complementary to target mRNAs, and these variants exhibit higher efficiency in directing human RNase P to cleave the target mRNAs than those derived from nature RNAs in vitro. This chapter describes the procedure of using engineered EGSs for in vitro trans-cleavage of target viral mRNAs in cultured cells. Detailed information is focused on (1) generation and in vitro cleavage assay of the customized EGS variants and (2) stable expression of EGS and evaluation of its activity in inhibition of viral gene expression and growth in cultured cells. These methods should provide general guidelines for using engineered EGS to direct RNase P-mediated cleavage of target mRNAs in cultured cells.

Keywords

Engineered external guide sequence; Human RNase P; In vitro evaluation; Human cytomegalovirus (HCMV); Gene targeting

1 Introduction

Nucleic acid-based gene interference strategies represent powerful tools to unravel functions of genes and promising therapeutic agents for human diseases [1]. As one of the most abundant, stable, and efficient enzymes in cells, ribonuclease P (RNase P) is responsible for the 5' end maturation of all tRNAs by catalyzing a hydrolysis reaction to remove a 5' leader sequence from tRNA precursors (ptRNA) [2] (Fig. 1a). The extensive studies on RNase P recognition revealed that it recognizes the structure rather than the sequence of the substrates [3, 4]. According to this unique feature, any complex of two RNA molecules that resembles a ptRNA can be cleaved by RNase P (Fig. 1b). Thus, in principle, RNase P can be recruited to cleave an mRNA if the mRNA substrate forms a hybrid complex with a custom-designed sequence (external guide sequence or EGS) to resemble a ptRNA molecule [5, 6] (Fig. 1c).

Unlike other nucleic acid-based interference approaches such as antisense oligonucleotides and RNAi [7], EGS-based technology is unique in inducing endogenous RNase P for targeted cleavage. Importantly, the RNase P-mediated cleavage is highly specific and does not generate nonspecific “irrelevant cleavage” that is observed in RNase H-mediated cleavage induced by conventional antisense phosphothioate molecules [8, 9]. EGS RNAs derived from natural tRNA sequences have been shown to be effective in blocking gene

expression both in bacteria and mammalian cells [6, 8, 10–13]. Moreover, in vitro selection procedure has been exploited to increase the targeting activity of EGSs in directing human RNase P to cleave an mRNA, which can lead to better efficacies of the EGSs in inducing RNase P-mediated inhibition of the expression of the target mRNA in cultured cells [14, 15]. Therefore, RNase P complexed with EGS represents a novel and promising nucleic acid-based gene interference strategy for specific inhibition of target mRNAs [15].

Human cytomegalovirus (HCMV), a human herpes virus, is a common opportunistic pathogen and the leading cause of congenital infections associated with mental retardation in newborns and can cause serious clinical manifestations in immunocompromised individuals, including AIDS patients and transplant recipients [16]. The emergence of drug-resistant strains of HCMV has posed a need for the development of effective antiviral approaches for the treatment and prevention [17]. In previous studies, an engineered EGS variant, which was used to target mRNA encoding the protease of HCMV, exhibited higher activity in inducing human RNase P to cleave the mRNA in vitro than the EGS derived from a natural tRNA [15]. These results demonstrated the feasibility of using engineered EGS as potential therapeutic agent against HCMV as well as other viruses.

In this chapter, detailed protocols involved in the in vitro generation of efficient EGS against protease (PR) mRNA of HCMV are described. The series of experiments undertaken to down-regulate the expression of the PR mRNA by EGS are (1) generation of EGS and target PR mRNAs in vitro, (2) determination of the efficiency of RNase P-mediated degradation of the target mRNA in the presence of the selected EGSs by in vitro cleavage assay, (3) stable expression of EGS in cultured cells, and (4) evaluation of EGS activity in inhibition of viral gene expression and growth in cultured cells.

2 Materials

2.1 Reagents and Solutions

1. β -Mercaptoethanol.
2. Dulbecco's modified Eagle's medium (DMEM).
3. Nu-serum, fetal bovine serum (FBS).
4. Neomycin.
5. Deoxynucleotide 5' triphosphates (dNTPs) and nucleotide 5' triphosphates (NTPs).
6. Diethylpyrocarbonate (DEPC)-treated H₂O.
7. [³²P]-labeled nucleotides.
8. 1× Denhardt's solution: 0.1 % Bovine serum albumin (BSA), 0.1 % polyvinylpyrrolidone, 0.1 % Ficoll, 0.1 % SDS, and 200 µg/mL denatured salmon sperm.
9. 20× Standard saline citrate (SSC): 3 M NaCl, 0.3 M trisodium citrate.
10. 10× TBE: 0.89 M Tris–borate, 10 mM EDTA.

11. 2× RNA dye solution: 8 M urea, 20 mM EDTA, 0.25 mg/mL bromophenol blue, 0.25 mg/mL xylene cyanol FF (XCFF).
12. TNT: 10 mM Tris-HCl, pH 7.0, 150 mM NaCl, 0.05 % Tween-20.

2.2 Enzymes, Reaction Buffers, and Kits

1. 10× PCR buffer: 25 mM MgCl₂, 10 mM dNTPs.
2. Buffer A (cleavage buffer): 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 100 mM NH₄Cl.
3. Buffer B (binding buffer): 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 10 mM NaCl, 10 mM MgCl₂, 3 % glycerol, 0.1 % xylene cyanol.
4. Annealing buffer: 10 mM Tris-HCl, pH 7.5, 10 mM KCl.
5. Prehybridization buffer: 6× SSC, 0.05 % sodium pyrophosphate, 2× Denhardt's solution.
6. *Taq* DNA polymerase (Perkin-Elmer).
7. T7 in vitro transcription kit (Promega).
8. RNA isolation kit (SurePrep™ kit, Fisher Bioreagents).
9. Mammalian transfection kit (GIBCO/BRL).
10. ECL Western blot detection kit (GE Healthcare).
11. Random primed Labeling kit (Roche).

2.3 Virus, Cells, Plasmids

1. HCMV (Strain AD169, ATCC).
2. Murine PA317 cells (amphotropic retrovirus packaging cell line).
3. Human foreskin fibroblasts (HFF).
4. Human U373MG cells (ATCC).
5. LXSJ (retroviral vector).

3 Methods

3.1 Construction of DNA Sequences Coding for EGS and PR mRNAs

Here we generate active EGS variants that can be used to effectively target mRNA. As described previously [14], by using the in vitro selection procedure to isolate EGS RNA variants, we chose variant 125 for this study. By covalently linking the EGS domain of C125 to the targeting sequences that are complementary to the PR mRNA, we constructed EGS PR-125. Another EGS, PR-SER, which was derived from the natural tRNA^{Ser} sequence, was also constructed in a similar way. The DNA sequences coding for the EGS were synthesized by PCR amplification, using construct plasmid as the template and were cloned under the control of the T7 RNA polymerase promoter.

1. To generate PR-SER, construct pTK112 DNA [12] was used as the template and the 5' and 3' primers were oligoPR31 (5'-GGAATTCTAATACGACTCACTATAGGTAA CGCGCCGGGTGCGGTCTCC-3') and oligoPR32 (5'-AAG CTTTAAATGCTCTCCGC AGGATTTGAACCTGCGCG CG-3'). To generate PR-C125, construct Ptkc125 DNA was used as the template and the 5' and 3' primers were oligoPR41 (5'-GGAATTCTAATACGACTCACT ATAGGTTAACGCG CCGGGCAGCTACTAGC AG-3') and oligoPR42 (5'-AAGC T T T A A A T G C T C T C C G C A G G C T C C G A A C T GCTAGTAG-3'). The DNA sequences coding for EGS PR-SER-C and PR-C125-C were derived from those for PR-SER and PR-C125, respectively, and contained point mutations (5'-TTC-3' → AAG) at the three highly conserved positions in the T-loop of these EGSs.
2. The DNA sequence that encodes substrate pr39, which contained a PR mRNA sequence of 39 nucleotides, was constructed by annealing oligonucleotide AF25 (5'-GGAATT CTAATACGACTCACTATAG-3') with sPR (5'-CGGGATC CGCAGCGCCGGCTGG AGAGCGAGAGGCCGG CCTAT AGTGAGTCGTATTA-3').
3. For a 100 µL PCR reaction, mix the following: 10 µL of 10× PCR buffer; 6 µL of 25 mM MgCl₂; 8 µL each of 10 mM dNTP; 20 pmol of pTK112 DNA templates; 400 pmol each of PR31 and PR32 oligomer; and 2.5 U *Taq* DNA polymerase.
4. PCR amplification program: Denaturing for 2 min at 94 °C; 30 cycles of 94 °C for 1 min, 47 °C for 1 min, and 72 °C for 1 min; final extension for 10 min at 72 °C.
5. The PCR DNA products are separated in 5 % polyacrylamide gels under non-denaturing conditions and are purified and used as the template for the next in vitro transcription step (*see Note 1*).

3.2 Generation of EGS and PR mRNAs by In Vitro Transcription

1. The gel-purified PCR DNA products are used as templates for the in vitro transcription of EGSs. A 100 µL in vitro transcription reaction mix is prepared by adding the following: 20 µL of 5× in vitro transcription buffer; 10 µL of 100 mM DTT; 5 µL each of 10 mM ATP, GTP, CTP, and UTP; 7 µL of PCR-generated DNA templates (~10 µg DNA); 1.5 µL of RNasin RNase inhibitor; 1.5 µL of T7 RNA polymerase.
2. Incubate at 37 °C for at least 16 h, stop the reaction by adding 100 µL of denaturing dye, and load on 8 % denaturing gels containing 7 M urea.
3. Visualize the full-length RNA products by autoradiography, and extract RNA (*see Note 2*) from the excised gel slice by the crush-soak method using DEPC-treated water (*see Note 3*).
4. The yield of the in vitro RNA synthesis is determined either by measuring the concentration of the RNA using a spectrophotometer or by quantitation of the total radioactivity by using a STORM 840 PhosphoImager.

5. Synthesize labeled PR mRNA as described above, except using 30 μCi α -[^{32}P]-GTP and 1 mM GTP instead of 10 mM GTP in the reaction.

3.3 In Vitro Cleavage Assay for Substrate pr39

1. Human RNase P was prepared from HeLa cellular extracts as described previously [18].
2. The EGSs and [^{32}P]-labeled pr39 were incubated with human RNase P at 37 °C in a volume of 10 μL for 45 min in buffer A.
3. Cleavage products were separated in denaturing gels and analyzed with a STORM840 PhosphorImager (GE Healthcare).

3.4 Measurement of K_d for the Target RNA by EGSs

To determine the stability of EGS-mRNA complex, equilibrium dissociation constant (K_d) for the target RNA by EGS variants was measured.

1. Prepare a 5 % polyacrylamide gel with a 30:1 weight ratio of acrylamide to *N,N'*-methylene bis-acrylamide in 36 mM Tris base/64 mM HEPES, pH 7.5, 10 mM MgCl_2 , and 0.1 mM EDTA. Run the gel in the same buffer at constant power, and maintain the temperature of the gel at 37 °C.
2. Dilute EGS to varying concentrations, ranging from 0.1 to 1,000 nM.
3. Mix 10 μL EGS dilutions at $2\times$ final concentration with 6 μL ^{32}P -labeled target mRNA (1,000 cpm and 0.1 nM). Heat at 80 °C for 3 min, and add 4 μL of $5\times$ binding buffer.
4. Incubate the samples at 37 °C for 10 min (to reach an equilibrium). Load samples onto the pre-warmed gel and run at a constant temperature of 37 °C immediately (see Note 4).
5. Dry the gel and quantitate free target RNA and bound RNA at each concentration of EGS on a PhosphorImager. The dissociation constant of an EGS is determined by inspection of the gel midpoint, where $\text{RNA}[P_{\text{free}}] = [P_{\text{bound}}]$. $K_d = [E_{\text{total at midpoint}}] - 1/2[P_{\text{total}}]$ (here $[P]$ and $[E]$ symbolize the target RNA and the EGS, respectively).

3.5 Kinetic Analyses of the Cleavage Reactions with EGSs

Multiple turnover kinetic analyses to determine the values Michaelis constant (K_m) and the maximum velocity (V_{max}) of the enzymatic reactions were carried out.

1. Mix varying amounts of ^{32}P -labeled target mRNA with an excess amount of EGS RNA in buffer A, and calculate the concentration of target RNA-EGS complex in each mixture based on the dissociation constants (K_d).
2. Perform standard RNase P reactions for 0.5, 1, 2, 5, and 10 min.
3. Extrapolate an initial rate of RNase P reaction for each concentration of RNA-EGS complex.

4. Determine the values of K_m and V_{max} using Lineweaver–Burk plots (double-reciprocal plots) [14].

3.6 Stable Expression of EGS RNAs in Human Cells

The DNA sequence coding for PR-C125 and PR-SER EGS was sub-cloned into retroviral vector LXSXN and was placed under the control of the small nuclear U6 RNA promoter [6, 19, 20].

1. To construct cell lines that express variant EGS RNAs, amphotropic packaging PA317 cells [21] were cultured in 6-well plates 1 day before transfection. When cell confluence was about 80 % (about 16 h after cell plating), transfect cells with 10–20 µg following retroviral vector DNAs (LXSXN-PR-SER, LXSXN-PR-SER-C, LXSXN-PR-C125, LXSXN-PR-C125-C) by using the mammalian transfection kit (GIBCO/BRL).
2. 48 h post transfection, collect culture supernatants (about 3 mL) and infect U373MG cells.
3. 1.5 mL of the collected retroviral stock was used to infect U373MG cells. The infected cells were incubated for 4–12 h with occasional shaking (*see Note 5*), and then the inoculums were replaced with fresh DMEM supplemented with 10 % FBS.
4. 48–72 h post transfection, cells were incubated in culture medium that contained 600 µg/mL of neomycin.
5. Neomycin-resistant cells were selected and cloned in the presence of neomycin for 2 weeks. Cells infected with retrovirus were subsequently split sparsely over ten cultured flasks and placed under neomycin to select for cloned EGS-expressing cell lines (*see Note 6*). Aliquot and freeze the selected cells for long-term storage in liquid nitrogen or for use in further studies.

3.7 Northern Analysis of the Expression of EGS RNAs in Cultured Cells

1. Wash the infected cells with PBS; both nuclear and cytoplasmic RNA fractions were isolated using SurePrep™ kit (Fisher Bioreagents). RNA concentration was tested by Bio-spectrophotometer (*see Note 7*).
2. 10 µg of RNAs are loaded onto a 2.5 % formaldehyde agarose gel with 45 mL of formaldehyde and 50 mL of 5× Northern buffer. RNAs are separated by running the gel at a constant voltage.
3. After washes of the gel with deionized water, transfer the RNAs onto a nitrocellulose membrane.
4. The transfer sandwich is set up in a large glass dish filled with 1 L 20× SSC solution from the bottom up: a long glass plate, a half sheet of Whatman 3 mm paper, three pieces of Whatman 3 mm paper (larger than the gel), the gel, a saran wrap cut with a window to expose gel, a piece of nitrocellulose membrane wetted with RNase-free water (larger than the gel), five sheets of Whatman 3 mm (the same size as the membrane), a stack of paper towels of about 4 cm at height, a

glass, and a weight (1 kg). At each step, air bubbles are trapped by gently rolling a test tube over the last layer added. After overnight transferring, the membrane is rinsed three times with deionized water for 10 min. Then the membrane is placed on top of a piece of Whatman 3 mm paper and baked in an oven at 80 °C for 1.5 h.

5. The nitrocellulose membrane is pre-hybridized for 4 h and hybridized for 16 h with the ³²P-radiolabeled DNA probes at 65 °C in the hybridization buffer.
6. Wash the membrane with 2× SSC, 1× SSC, and 0.5× SSC (containing 0.1 % SDS).
7. Expose the membrane and analyze with a STORM840 PhosphorImager.

3.8 Determination of RNase P-Mediated Inhibition of Viral Gene Expression and Growth in Cultured Cells

3.8.1 Viral Infection

1. 1×10^6 Cells were either mock-infected or infected with HCMV at a multiplicity of infection (m.o.i.) of 0.5–5 in an inoculum of 1.5 mL of DMEM supplemented with 1 % fetal calf serum.
2. The inoculum was replaced with DMEM supplemented with 10 % (v/v) FBS after 2-h incubation with cells.
3. The infected cells were incubated for 4–72 h before harvesting for isolation of viral mRNA or protein.
4. To measure the levels of viral immediate-early (IE) transcripts, some of the cells were also treated with 100 µg/mL cycloheximide prior to and during infection.

3.8.2 Northern Blot Analysis

1. Wash the infected cells with PBS; RNA fractions were isolated as described in Subheading 3.7 (**step 1**).
2. For detection of viral mRNAs, the RNA fractions were separated in 1 % agarose gels that contained formaldehyde, transferred to a nitrocellulose membrane, hybridized with the ³²P-radiolabeled DNA probes that the HCMV DNA sequences, and analyzed with STORM840 PhosphorImager.

3.8.3 Western Blot Analysis—Western analyses are performed to determine the expression level of HCMV PR proteins.

1. Wash the cells twice with 5 mL PBS, and spin down cells with $3,000 \times g$ at 4 °C for 5 min.
2. Suspend cell pellets in 50–100 µL cold PBS, followed by adding the same volume of 2× disruption buffer.
3. Vortex the mixture for 1 min, followed by three times sonication on ice. Each lasts for 20–30 s.
4. Boil the sample for 5 min before loading on SDS polyacryl-amide gel.

5. Load 50 µg of proteins onto either 7.5 or 9 % SDS polyacrylamide gel cross-linked with *N,N'*-methylenebisacrylamide with a stacking layer of 4.5 % acrylamide/bisacrylamide. Separate the proteins by running the gels at a constant power setting.
6. The proteins are transferred onto a nitrocellulose membrane (*see Note 8*), using an electrophoretic transfer apparatus with a constant current of 150 mA for 2 h.
7. Incubate the nitrocellulose membrane with TNT buffer plus 2.5 % skim milk for 1 h in an orbital shaker (blocking step).
8. After blocking, incubate the membrane with primary antibody at a dilution of 1:500 in TNT supplemented with 1.25 % of skim milk for 1 h.
9. Wash the membrane with TNT buffer three times and 5 min for each wash.
10. Incubate the membrane with secondary antibody diluted at 1:1,000 dilutions for 1 h, followed by three washes of the membrane with TNT buffers and 5 min for each wash.
11. Incubate with ECL substrates for 1 min at RT, expose membrane, and develop film.

3.8.4 Analysis of Viral Growth by Plaque Assay

1. 5×10^5 of EGS-expressing cells are infected with HCMV at an m.o.i. of 0.5–2.
2. Incubate the cells with DMEM for 1.5 h, wash with PBS, followed by adding 0.5 mL fresh DMEM supplemented with 10 % Nu-serum.
3. The cells and medium are harvested at 1, 2, 3, 4, 5, 6, and 7 days post infection.
4. Viral stocks are prepared by adding an equal volume of 10 % (v/v) skim milk, followed by sonication.
5. Prepare tenfold serial dilution of the viral stock in 2 mL of DMEM for each dilution.
6. Infect 1×10^5 HFF cells in a 6-well plate with 1 mL of viral dilutions, and incubate for 2 h.
7. Wash the cells with DMEM, and overlay the cells with fresh 1 % agarose and DMEM containing 10 % Nu-serum in a 1:1 ratio.
8. Count the number of viral plaques 10–14 days after infection.
9. Plaque-forming unit (PFU) is determined by the highest viral dilution that yields plaque.

3.8.5 Determination of the Level of Intracellular HCMV Genome—To determine the antiviral mechanism resulting from the EGS-directed cleavage, we carried out a series of experiments to investigate whether EGS-based inhibition of PR expression affects viral genomic DNA replication as well as viral DNA encapsidation. The encapsidated viral DNAs would be resistant to DNaseI digestion, whereas those that are not packaged in the capsid would be susceptible.

1. The level of intracellular viral genomic DNA was determined by PCR detection of the sequence of immediate-early IE1 sequence, using the human β -actin sequence as the internal control. The 5' and 3' primers for detecting the IE1 sequence were CMV3 (5'-CCAAGCGGCCTCTGATAACCAAGCC-3') and CMV4 (5'-CAGCACCATCCTCC TCTTCCTCTGG-3'), respectively. The 5' and 3' primers used to amplify the β -actin sequence were actin5 (5'-TGACGGGGTCACCCA CACTGT GCCCATCTA-3') and actin3 (5'-CTAGAAGCATTGCGGT GGCAGATGG AGGG-3'), respectively.
2. 5×10^5 cells grown on 6-well plates were mock-infected or infected with HCMV.
3. After 1.5-h incubation at 37 °C, the inoculum was removed and the cells were further incubated and harvested at 72–96 h post infection.
4. Total and encapsidated (DNase I-treated) DNAs were isolated essentially as described [22] and used as the PCR DNA templates.
5. The PCR amplification consisted of 20 cycles (*see Note 9*) with denaturation at 94 °C for 1 min, followed by primer annealing at 47 °C for 1 min and extension at 72 °C for 1 min. The last cycle was again an extension at 72 °C for 10 min.
6. The amplified HCMV DNA (481 bp) and actin sequence (610 bp) were separated on 4 % non-denaturing polyacrylamide gels.

4 Notes

1. Although there are several methods available for oligonucleotide purification, gel purification is suggested here, since the unused primers can be removed in this process.
2. It is recommended to warm up the RNA samples at 37 °C for at least 5 min before loading on the gel. To avoid urea precipitation, flush the well ahead of loading samples.
3. DEPC-treated H₂O is recommended for the entire gel-running process to avoid mRNA degradation.
4. Maintaining the gel at 37 °C is critical to obtain accurate values of K_d ; thus, pre-running the gel in the same buffer at constant power is suggested in this step.
5. To increase the efficiency of retroviral infection in targeted cells, medium supplemented with 8 μ g/mL polybrene is recommended. Alternatively, multiple rounds of infection can also be carried out to improve the efficiency.
6. More than ten clones are selected in this step, and most of them were found to express high level of ribozymes.
7. RNA fractions are extracted from nucleus and cytoplasm and tested by Northern blot separately, since the EGS RNA expressed by U6 promoter is primarily localized in the nucleus.

8. The nitrocellulose membrane should be soaked in distilled water for 2 min, followed by placing into the transfer buffer, and allowed to soak for 5 min. Transfer of protein from the gel to the membrane should be performed at 4 °C.
9. PCR cycles and other conditions were optimized to assure that the amplification is within the liner range. We also generated a standard (dilution) curve by amplifying different dilutions of the template DNA. The plot of counts for both HCMV and β -actin versus dilutions of DNA did not reach a plateau for the saturation curve under certain conditions, indicating that quantitation of viral DNA could be accomplished.

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Fig. 1. Substrates for ribonuclease P (RNase P). **(a)** Schematic representation of a natural substrate (precursor tRNA, ptRNA) and can be cleaved by RNase P. **(b)** A hybridized complex of a target mRNA and an external guide sequence (EGS) that resembles the structure of a ptRNA. **(c)** Results from **(b)** achieved deleting the anticodon domain of the EGS, which is indispensable for EGS-targeting activity [18]. The site of cleavage by RNase P is indicated with an *arrowhead*. The sequence of the target mRNA around the targeting site is shown in *purple*, and the EGS sequence is shown in *green*