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RESEARCH PAPER

Engineering of Thiamine Pyrophosphate Fluorescent Biosensors Based on Ribozyme Switches in Mammalian Cells

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Abstract: As the main active form of intracellular thiamine (vitamin B1), thiamine pyrophosphate (TPP) is an essential cofactor in oxidative metabolism of the sugars, fatty acids and amino acids. By now, numerous TPP-dependent artificial riboswitch systems have been developed to regulate target gene expression but are limited in bacteria, fungi or plant cells. Herein, the activating (switch-on) and inhibiting (switch-off) TPP-dependent hammerhead ribozyme switches, inspired by the previously reported structures of prokaryotes screening, were investigated in mammalian cells. Such ribozyme switches were inserted into the 3′-UTR of the enhanced green fluorescence protein (EGFP) gene through overlap extension PCR cloning and were further transfected into HEK293 cells at increasing concentrations of TPP. The EGFP gene-regulatory ability was analyzed with fluorescent microscope and flow cytometry. These resultant TPP-inducible gene regulation devices showed obvious ligand dose-dependency and excellent specificity. Upon the addition of 150 μM TPP, two "switch-on" and one "switch-off" constructs demonstrated 3.1-fold or 1.9-fold increment and 2.3-fold reduction of EGFP level, respectively. The ligand-responsive ribozyme switches, by transforming the change of TPP concentration into the visual reporter genetic expression in cells, enable efficient development of label-free, noninvasive and high specific biosensors in living mammalian cells.

Key Words: Thiamine pyrophosphate ribozyme switches; Enhanced green fluorescence protein; Gene expression regulation; Fluorescent biosensors; Mammalian cells

1 Introduction

The artificial aptazyme-based riboswitches are an emerging class of noncoding genetic control elements that have become a powerful tool for gene regulating in recent years. The most common aptazyme riboswitch consists of an aptamer domain and an expression platform[1]. As a *cis*-acting element, it can sense a chemical stimulus and induce a structural change or an irreversible self-cleavage with no need of protein co-factors, thus affecting transcription elongation, translation initiation or mRNA stability of genes[2–6]. A growing number of aptamers, which can specifically recognize targets such as small molecules, proteins, peptides, amino acids, antibiotics and metal ions[7–10], were obtained with systematic evolution of ligands by exponential enrichment (SELEX) technique[11]. Compared with natural ribozyme switches, these artificial riboswitches have characteristics of higher gene specificity, structural flexibility and tight gene expression control ability. So, rational design of ligand-responsive ribozyme switches might be available for tuning the variation of ligand concentration into visual reporter genetic expression in cells, which enables an efficient development of in situ and visual

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detection system in living mammalian cells. Furthermore, the combination applications of synthetic biology and logic gate systems would create more realistic and complex systems, which extends the potential applications of ribozyme switch-based biosensors, thus showing brighter future for *in vivo* sensing, gene therapy and biological processor in eukaryotic cells[13].

As a thiamine (vitamin B1) derivative and an essential cofactor in oxidative metabolism of the sugars, fatty acids and amino acids, thiamine pyrophosphate (TPP) plays an important role in cell metabolism, growth and proliferation. TPP ribozyme switch is the unique natural RNA switch found both in prokaryotes and eukaryotes. They have been found in the plants and fungi where they appear to operate by alternative pre-mRNA splicing of the intron[14–17]. This accurate and effective gene regulation allows TPP ribozyme switch to have high sensitivity and specificity to the ligand[18–20], thus avoiding being falsely activated by other components. By now, while a number of artificial riboswitch systems have been developed to regulate target gene expression in bacteria, fungi, alga cells or in vitro[21–24], these devices have been reported to respond to only several ligands, including theophylline, tetracycline and metal ions in mammalian cells[25,26]. As an important eukaryotic cell metabolic intermediate, with non-toxic characteristic and low background concentration in the cells, TPP is essential for developing artificial ribozyme switches for gene regulation in mammalian cells.

In prokaryotic cells, the allosteric riboswitch is located at 5' untranslated region (UTR) of the target gene whose ribosomal binding site (RBS) sequences can be released or sequestrated by the ligand-induced change of mRNA secondary structures, thus leading to gene expression "turning on" or "turning off". On the contrary, being without RBS sequences, the eukaryotic mRNA is regulated by 5'-cap and 3'-poly (A) tail elements. Therefore, TPP-activated riboswitches increase pre-mRNA cleavage and hence decrease gene expression, while TPP-inhibited riboswitches shut off self-cleavage of the mRNA and increase gene expression upon the external addition of TPP[28,29].

At present, the most popular approach to design an aptazyme-based riboswitch is rationally engineering an aptamer into a ribozyme, then screening with a high throughout method after integration into mRNA[30,31]. However, the screening which is applicable in bacteria or yeast might not work efficiently in mammalian cells with complicated and unpredictable cell environment. Therefore, the function validation in mammalian is necessary.

According to the previous report, we present the "on" or "off" TPP-dependent hammerhead ribozyme switches (Fig.1), which differ in six oligonucleotide sequence between aptamer and hammerhead shape ribozymes (HHR)[21,23]. Then they were inserted into the 3′-UTR of enhanced green fluorescence protein (EGFP) gene. The regulation ability of two types of ribozyme switches was monitored by transient transfection into human embryonic kidney 293 cells. These ligand-responsive ribozyme switches, by transforming TPP concentration change into the visual reporter genetic expression *in vivo*, enables efficient development of label-free, noninvasive and high-specific biosensors in living mammalian cells.

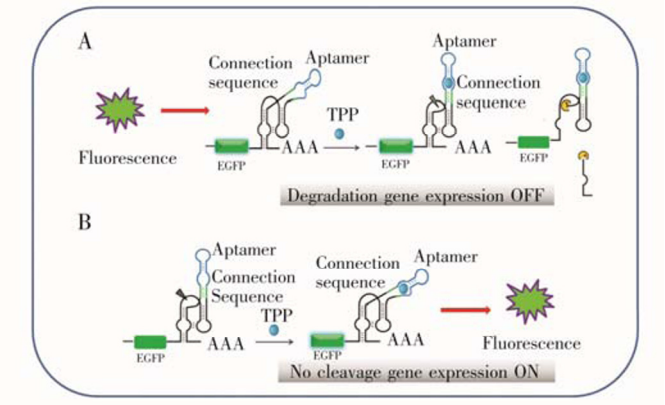

Fig.1 Mechanism of TPP hammerhead ribozymes as the regulatory components for EGFP gene expression in eukaryotes with (A) switch-off or (B) switch-on strategies

(A) "switch-off" ribozymes increase pre-mRNA cleavage in the presence of TPP, which results in the decrease of EGFP gene expression. (B) "switch-on" ribozymes shut off self-cleavage of pre-mRNA upon the external addition of TPP, which leads to the increase of EGFP gene expression

2 Experimental

2.1 Instruments and reagents

Eppendorf mastercycler nexus PCR (Eppendorf Company, Germany), Nikon ECLIPSE TI inverted fluorescence microscope (Nikon Company, Japan), BD Accuri C6 flow cytometry instrument (BD Company, USA) were used in this study.

Focal sulfuric acid thiamine (> 95.0%) was purchased from Sigma, USA. Theophylline (> 99.0%) was purchased from Beijing technology Company, LTD. DpnI enzyme (50 U μL^{-1}) was purchased from NEB Company, USA. T4 ligase (5 U μL^{-1}), lipofectamine 2000 transfection reagent and DMEM medium were purchased for Invitrogen Company, USA. Calf serum was obtained from Gibco, USA.

2.2 Plasmid construction

The hammerhead shape ribozymes (HHR) sequence from *Schistosoma mansonii* (Fig.2B) was inserted into 3'-UTR of EGFP gene carried by plasmid pEGFP-N_1 to construct the plasmid HHR without TPP aptamer (pEGFP-HHR). The primer and template sequences are listed in Table 1. Plasmids were constructed by using overlap PCR cloning: 94 °C, 2 min; 94 °C, 30 s; 60 °C, 30 s; 72 °C, 1 min; 25 cycles; 72 °C, 10 min. PCR reaction system: 5 μL 10 × Phusion DNA polymerase buffer; 4 μL 10 mM dNTP; 2 μL 10 μM primer F; 2 μL