Development of a Novel Functional Gene Discovery System by Hybrid-Ribozyme Libraries in the Post Genome Era

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1 Introduction

Hammerhead ribozymes are small RNA molecules that can catalyze cleavage of an RNA both in cis and in trans. Since these ribozymes can be designed to cleave any RNA substrate that contains an NUX triplet (where N can be any nucleotide and X can be C, A or U), they have been used successfully to knock out the intracellular expression of a variety of specific viral and cellular targets [5, 2]. However, the efficiency of ribozyme-mediated cleavage in vivo is not always as high as anticipated or required. In vivo, the activities of ribozymes depend on their access to the cleavage site in the target RNA. To solve this target problem and to improve the efficiency of ribozymes in vivo, we have created a ribozyme with the ability to access any target site and to cleave at a specific site. This was accomplished by combining the cleavage activity of the hammerhead ribozyme with the unwinding activity of the endogenous RNA helicase. Then, we demonstrate that ribozymes of this type are able to cleave the target mRNA at any chosen site, regardless of the putative secondary or tertiary structure in the vicinity of the target site, and thus, they can be used for rapid identification of functional genes in the post-genome era.

The sequence of the human genome is available and it is important to identify functional human genes and to clarify their functions in the signal transduction network. In this study, we demonstrate that a rapid identification of functional genes is possible using the RNA helicase-associated hybrid-ribozyme libraries with randomized substrate-binding arms. When the phenotype of cells changes upon introduction of a ribozyme library, genes that are responsible for the changes in phenotype can be identified by sequencing the active ribozyme clones. Since, when this method is used, inhibition of the expression of a particular gene is reflected by a change in a particular phenotype, the method allows the easy and rapid identification of functional genes. Thus, our gene discovery system using hybrid-ribozyme libraries should be useful for the rapid identification of functional genes in the post-genome era.

2 Method and Results

2.1 Construction of Novel RNA-Protein Hybrid Ribozyme Libraries

For construction of hybrid-ribozyme libraries, we added a sequence of an RNA helicase binding motif (RBM) to the 3' end of a tRNAVal-driven ribozyme [3, 6, 7]. In order to test the efficacy of hybrid
ribozymes, we designed four hybrid ribozymes and conventional ribozymes aimed at specific targets. Three ribozymes, namely, Rz1, Rz2 and Rz3, were designed to target inaccessible sites that are located within the stable stem-structure. By contrast, Rz4 was designed, as a control, to target a relatively accessible site located in a loop region of the target mRNA.

To examine whether RNA helicase would associate with tRNAVal-Rz-RBM in vitro, we performed a biotin-streptavidin “pull-down” assay using biotin-labeled tRNAVal-Rzs or tRNAVal-Rz-RBMs. As a result, tRNAVal-Rzs transcripts without a RBM sequence did not bind the RNA helicase. These results indicated that the endogenous RNA helicase associated with tRNAVal-Rz-RBM in vitro. Next, to examine whether tRNAVal-Rz-RBM-protein complexes can cleave target inaccessible sites, we performed in vitro cleavage assay by these ribozyme-protein complexes. As a result, RBM-unconnected Rz1, Rz2, Rz3 did not unwind the duplexes and, thus, they were unable to cleave the substrate. These results clearly demonstrate that they were able to cleave the target mRNA at any chosen site, regardless of the putative secondary or tertiary structure in the vicinity of the target site.

Then, we generated two libraries of $1 \times 10^7$ different RBM-connected or unconnected ribozymes with randomized substrate-binding arms. We introduced a total of 20 random nucleotides into the target recognition sites of the ribozyme libraries.

2.2 Functional Gene Discovery Using Hybrid-Ribozyme Libraries

The sequence of the human genome has become available and it will be extremely valuable to have methods for the rapid identification of important genes [3, 4, 6]. Since our hybrid ribozymes can attack any site, they can attack any mRNA. If libraries of hybrid ribozymes with randomized binding arms are introduced into cells, the genes associated with any changes in phenotypes can be readily identified by sequencing of the specific ribozyme clone [3, 4, 6].

This procedure was used to establish a novel functional gene screening system for the signal pathway of Fas or TNF-a-induced apoptosis using the randomized Rz-RBM expression libraries. In this system, we randomized ten nucleotides in each substrate-binding arm of Rz-RBM. After treatment of the randomized Rz-RBM introduced cells with the Fas specific antibodies or TNF-a, cells that survived were collected and a respective genomic DNA was isolated from each clone. The sequences of the active ribozymes from the genomic DNAs were determined with a DNA sequencer. Finally, the target genes of these ribozymes were located and identified with the BLAST search program [1]. Then we identified many pro-apoptotic genes and novel genes using this strategy. It should be emphasized that, in the absence of the RBM, we would not have identified many genes in our screening. Moreover, we identified functional genes in other signal pathways such as cancer genetics or neural development. Thus, our gene discovery system using hybrid-ribozyme libraries should be useful for the rapid identification of functional genes in the post-genome era.

References
