0273-2289/95/5401-03—0057$05.00

Colocalizing Ribozymes with Substrate RNAs to Increase Their Efficacy as Gene Inhibitors

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ABSTRACT

The ability to target ribozymes to specifically cleave viral RNAs in vitro has led to much speculation about their potential therapeutic value as antiviral agents in vivo. To transfer a ribozyme's potential as an antiviral agent from test tubes to cells and organisms successfully, the characteristics that distinguish these settings must be considered. In vitro, ribozymes and substrate RNAs freely diffuse in solution in test tubes, and trans-cleavage reactions are dependent on a diffusive step. In eukaryotic cells, by contrast, many RNAs do not appear to diffuse freely. Instead, they appear to be highly compartmentalized and actively sorted to specific cellular locations. Such RNA trafficking may result in localization of substrate RNAs in a different compartment than ribozymes, which would effectively reduce substrate RNA availability to ribozymes and therefore limit the effectiveness of ribozymes as gene inhibitors.

Index Entries: Ribozyme; antiviral agent; gene inhibition; cellular localization; RNA trafficking.

INTRODUCTION

Certain RNA molecules can adopt three-dimensional conformations that allow them to perform enzymatic reactions. These RNA enzymes or ribozymes contain catalytic cores and active sites that allow them to catalyze cleavage and ligation reactions on specific substrate RNAs with rate enhancements similar to those achieved by many protein enzymes (for review, *see* 1 and 2). The ability to redesign ribozymes to cleave target

RNAs catalytically in trans, in vitro has led to much speculation about their potential usefulness as gene inhibitors in vivo. Five catalytic RNA motifs have been described that may be amenable for trans-cleavage of target RNAs in vivo: RNase P, the hammerhead ribozyme, the hairpin ribozyme, the hepatitis Δ virus ribozyme, and the group I intron. Several recent reviews discuss the activities and potential of in vivo applications of each of these ribozymes (*3–6*). Therefore, what follows is only a brief review of such information with some potential advantages and disadvantages associated with in vivo use of each type of ribozyme highlighted.

CLASSES OF RIBOZYMES

Of the five motifs, only RNase P cleaves multiple-substrate RNAs in trans in nature. This ribozyme recognizes and cleaves tRNA precursors to remove extra sequences from their 5′ ends (*7*). Forester and Altman (*8*) have demonstrated that RNase P from *E. coli* can be directed to cleave non-tRNA sequences in vitro by base pairing an antisense RNA to a target RNA, such that the trinucleotide sequence CCA hangs from the end of the duplex, and thus resembles a pre-tRNA substrate. Expression of antisense RNAs, which base pair to targeted RNAs in this manner, can potentially lead to RNase P-mediated destruction of target RNAs in vivo. In practice, such destruction would require the assembly of the antisense/target duplex RNA, followed by recognition of the complex and cleavage of the target RNA by endogenous RNase P. A major potential advantage of this strategy is that RNase P naturally performs similar reactions continually in eukaryotic cells. However, RNase P performs this reaction on pre-tRNAs, which probably reside in different nuclear compartments from that in which mRNAs are found and processed. Therefore, if RNase P is sequestered in intracellular locations essential for pre-tRNA processing, it may not have access to many targeted mRNAs.

The hammerhead (*9*), hairpin (*10*), and hepatitis Δ virus ribozymes (*11*) are small cis-cleaving ribozymes that have been found mainly in plant virusoids and satellite RNAs or in a satellite RNA of human hepatitis B virus. Enzymatic versions of each of these ribozymes have been created that are capable of trans-cleaving novel substrates in vitro with multiple turnover (*12–17*). These ribozymes contain guide sequences that allow them to base pair with, and subsequently cleave, specific substrate RNAs in trans. Specific substrate sequence requirements are minimal for hammerhead and hairpin ribozymes, and they thus allow for the design of a particular ribozyme capable of cleaving a designated RNA by adjusting the length and composition of the ribozyme's guide sequence. Their small size and their malleability make these ribozymes vary attractive potential gene inhibitors. However, their eventual usefulness will depend on whether or not they can be adapted to cleave substrates efficiently in trans in the cellular milieu.

IN VITRO AND IN VIVO CLEAVAGE REACTIONS

The most thoroughly characterized ribozyme is the enzymatic form of the *Tetrahymena thermophila* group I intron discovered by Cech and colleagues (*18–20*). This ribozyme recognizes and cleaves after a specific hexanucleotide substrate sequence. Because a hexanucleotide sequence is expected to be present on many RNAs in vivo, use of this ribozyme as an in vivo gene inhibitor would probably be deleterious to cells. However, an important lesson can be learned from studying the detailed kinetic characterization of this ribozyme's in vitro trans-cleavage reaction by those wishing to employ ribozymes as in vivo gene inhibitors. Under $(k_{cat}/K_m)^s$ or single-turnover conditions, where the substrate concentration is subsaturating, the rate-limiting step for the cleavage reaction is the binding of substrate. Under k_{cat} or multiple-turnover conditions, when substrate is at saturating concentration, the release of the cleaved product is rate-limiting (*see 21* and *22* for a much more detailed discussion). Under neither condition is the actual chemical cleavage step rate-limiting. Therefore, to improve the in vitro catalytic efficiency of the ribozyme under k_{cat} or multiple-turnover conditions, the rate of product release must be increased. A mutant form of the ribozyme has recently been described that accomplishes this feat, despite the fact that the mutant's chemical step is slower than that of the wild-type ribozyme (*23*). Just as this kinetic framework allows one to try to improve ribozyme-mediated reactions logically in vitro, understanding what limits similar reactions in vivo should also allow one to attempt logically to increase the effectiveness of ribozymes as in vivo gene inhibitors. Only after we understand what limits ribozyme-mediated destruction of viral RNAs in vivo will we be able to predict accurately how useful ribozymes will be as antiviral agents.

How might the cellular setting influence ribozyme cleavage reactions? In vitro, ribozyme-mediated trans-cleavage reactions are dependent on a diffusive step (*2,21,22*). Under single-turnover conditions $(k_{cat}/K_m)^s$, cleavage can be made to proceed almost as fast as RNA duplex formation occurs in solution. In cells, in contrast, RNAs do not appear to diffuse freely. Rather, they appear to be highly compartmentalized and actively sorted to specific cellular locations (*24–27*). Such compartmentalization of viral RNAs in vivo may reduce their availability to ribozymes. Therefore, inside cells k_{on}, the rate of ribozyme and substrate association, will probably be greatly influenced by the localization and compartmentalization of the ribozyme and substrate RNAs. If ribozymes are sorted to different cellular sites than their substrates, then k_{on} may become extremely rate-limiting. For example, if a eukaryotic DNA polymerase did not encode a nuclear localization signal, then it would stay in the cytoplasm and would be unable to associate with the DNA replication machinery inside the nucleus. This extremely ineffective enzyme would be limited by the rate at which it found its way through the nuclear membrane. Alternatively, imagine a specially created ribozyme that under $(k_{cat}/K_m)^s$ condi-

tions in vitro, cleaves substrate RNAs with a $t_{1/2}$ = 1 min. If the substrate RNAs were encapsidated in liposome A and the ribozyme RNAs in liposome B before adding the RNAs to the test tube, and if the liposomes had an average half-life of 1 h, then the overall rate of the cleavage reaction would be greatly reduced, because the rate at which the RNAs were released from the liposomes would become rate-limiting. In both of these examples, k_{on} becomes extremely rate-limiting because the enzymes have been physically separated from their substrates. This situation may be common in cells, because large molecules are physically separated with respect to their functions in vivo. Therefore, if we are to employ biological macromolecules, such as ribozymes, as therapeutic agents, then it will almost certainly be necessary to take into account the fact that cells organize such molecules into specific, complex matrices. We should attempt to take advantage of the cell's propensity to compartmentalize such biological molecules in an ordered fashion so as to sort these therapeutic agents to desired sites in eukaryotic cells. This approach should increase the effectiveness of the agent by increasing its concentration at important intracellular sites and should reduce any side effects by allowing lower doses to be administered.

COLOCALIZING RIBOZYMES AND SUBSTRATES IN MAMMALIAN CELLS

The hypothesis that ribozyme-mediated trans-cleavage of a substrate RNA in vivo is limited by the rate at which substrate and ribozyme RNAs are colocalized within a cell has been tested by employing two retroviral vectors and taking advantage of the mechanism by which retroviruses sort their genomic RNAs in vivo (*28*). The transcripts generated from a *lac*Z containing retroviral vector were targeted for ribozyme-mediated cleavage within ecotropic packaging cells. Such *lac*Z transcripts have two distinct fates in such packaging cells. Some serve as mRNAs being translated to produce β-gal enzyme within these cells, whereas others serve as genomic RNAs and are packaged into viral particles budding from the surface of the cells. This latter process is mediated by the retroviral encapsidation machinery that recognizes the viral packing signal, Ψ, on the retroviral vector RNAs. Two hammerhead ribozymes designed to cleave the *lac*Z transcripts were cloned into and expressed from separate retroviral vectors. Ribozyme and mutant ribozyme containing vectors were transfected into an amphotropic packaging cell line, and the resulting virus was used to infect 10^4 *lac*Z vector containing packaging cells at a multiplicity of infection of 10. This approach was employed to avoid artifacts associated with clonal selection. Both anti*lac*Z ribozyme and *lac*Z substrate RNAs contain Ψ. Thus, ribozyme and *lac*Z containing genomic RNAs should be colocalized by the viral encapsidation machinery, whereas transcripts serving as mRNAs should not be colocalized. *Lac*Z viral titer

was reduced by 87–95% from anti*lacZ* ribozyme containing cells as compared to control and mutant ribozyme containing cells, whereas no reduction in β-gal enzyme activity within these cells was observed. Thus, colocalization of ribozymes with their substrates in the appropriate compartment within a eukaryotic cell appears to be important for the efficacy of the ribozyme as a gene inhibitor.

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