RNA targeting at any chosen site of interest by the novel RNA-protein hybrid ribozyme and the gene discovery based on the hybrid ribozyme libraries

H. Kawasaki,[a,b] T. Kuwabara,[b] and K. Taira[a,b]★

[a]Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, Hongo, 113-8656 Tokyo, Japan.
[b]Gene Function Research Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), Central 4, 1-1-1 Higashi, 305-8562 Tsukuba Science City, Japan.
Fax: 81 (298) 61 3019; 81 (358) 41 8828. E-mail: taira@chembio.t.u-tokyo.ac.jp

RNA targeting by the RNA-protein hybrid ribozymes, whose protein part can specifically bind to the RNA helicase, is described.

Key words: hammerhead ribozymes, metalloenzymes, maxizymes, hybrid ribozymes, genes.

Ribozyme activity *in vivo* depends on achieving high-level expression, intracellular stability, target co-localization, and cleavage site excess. At present, target site selection is problematic because of unforeseeable secondary and tertiary RNA structures that prevent cleavage. To overcome this design obstacle, the **R**NA helicase **B**inding **M**otif (RBM), *viz.*, an RNA motif that has the ability to interact with intracellular RNA helicases, was attached to our ribozymes so that the helicase-attached, hybrid-ribozymes would be produced in cells. We found that attachment of the RNA motif to our tRNA ribozyme leads to cleavage *in vivo* at the chosen target site independent of local RNA secondary or tertiary structure. Since the novel hybrid ribozymes can attack any site within mRNA, libraries can be made of the hybrid ribozymes with randomized binding arms and be introduced into cells. This novel ribozyme technology represents a new paradigm of powerful ribozyme technology and can enjoy many unique and exciting uses for various purposes in the post-genome era, including applications for discovery of novel functional genes associated with specific important phenotypes as well as for targeted elimination of expression of disease-causing genes *in vivo* in gene therapy approaches.

Hammerhead ribozymes are among the smallest catalytic RNAs. They are called "hammerheads" because of their two-dimensional structure. The sequence motif, with three duplex stems and a conserved "core" of two nonhelical segments that are responsible for the self-cleavage reaction (*cis*-action), was first recognized in the satellite RNAs of certain viruses. However, hammerhead ribozymes have been engineered in the laboratory to be able to act "in *trans*," and *trans*-acting hammerhead ribozymes consisting of an antisense section (stems I and stem III) and a catalytic core with a flanking stem-loop II section have been used as potential therapeutic agents and in mechanistic studies (Fig. 1).[1,2] Some ribozymes used in this work were developed as a result of studies to shorten the ribozyme.[3—9] Using a construct in which the substrate-binding region of the ribozyme is complementary to the target RNA (locations marked X—Y and N—M in the substrate-binding region), one can create molecular "scissors" that cleave the target RNA in a site-specific manner. When the catalytic core captures the catalytically indispensable Mg^{2+} ions, cleavage occurs only adjacent to the site with the triplet sequence NUX (where N is any base, and X is A, C, or U). In using the ribozyme in medical applications, ease of design and economics dictate that smaller size is preferable. Therefore, the design and construction of short ribozymes, namely, the minizymes, have been attempted by many investigators,[10—13] but unfortunately, such minizymes were found to have dramatically lower activities. However, we found that the minizyme completely lacking the stem-loop II region had activity essentially equivalent to the wild type.[3] Subsequent reaction kinetic analysis and NMR analysis indicated that the shortened ribozyme was essentially inactive as a monomer, but exhibited extremely high cleavage activity as a dimer. We initially called this ribozyme a "dimeric minizyme," but later renamed this structure "maxizyme" (see below). In addition, we also designed a heterodimeric system made of two different monomers, maxizyme left (MzL) and maxizyme right (MzR).[5—9] In this system, if MzL and MzR form a dimeric structure, the substrate will be cleaved.

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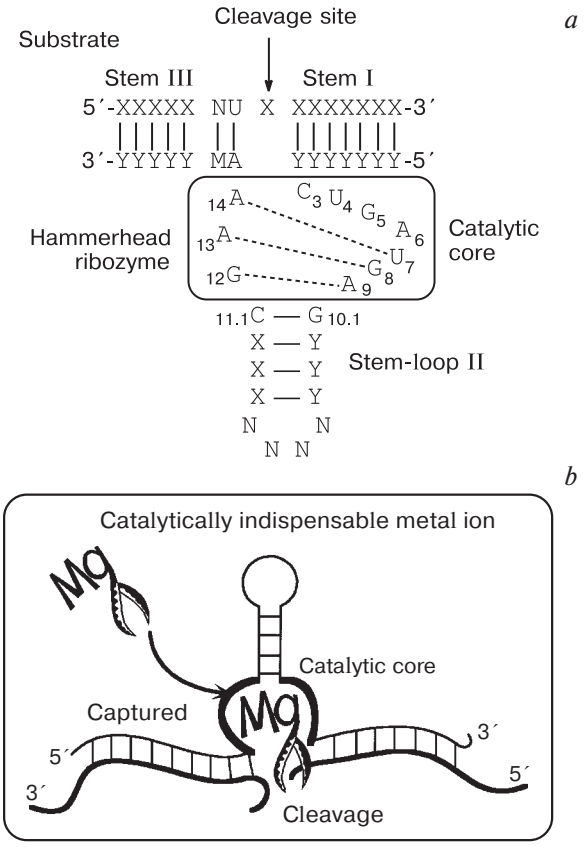

Fig. 1. Schematic representation of the secondary structure of the hammerhead ribozyme (*a*) and its mode of action (*b*).

The maxizymes described above were developed as a result of studies to shorten the hammerhead ribozyme. Because of the limitation for cleavable sequences on the target mRNA, in some cases conventional ribozymes fail to possess precise cleavage specificity. To overcome this problem, an allosteric version (a maxizyme that functions as a dimer) was developed that displayed activity and specificity *in vivo* (Fig. 2). The maxizymes, which function as dimers, have two substrate-binding regions. Such maxizymes form highly active dimers and successfully cleave various unwanted mRNAs both *in vitro* and *in vivo*.[4—9] We have taken advantage of the feature that maxizymes (a heterodimer MzL—MzR) can generate two different target sites (one complementary to the sequence of interest, *i.e.*, activator or inhibitor, and the other complementary to a cleavable sequence), to develop a system that can be used to inactivate gene expression. To achieve high substrate specificity, a maxizyme should be in an active conformation only in the presence of abnormal BCR—ABL junction, its conformation should remain inactive in the presence of normal ABL mRNA or in the absence of BCR—ABL junction. More than five custom-designed maxizymes have clearly demonstrated sensor functions, indicating that our technology might be broadly applicable in molecular biology and possibly in a clinical setting.

However, despite extensive efforts, the efficiencies of ribozymes *in vivo* usually are not always high enough to achieve the desired biological effect(s).[14] Successful gene

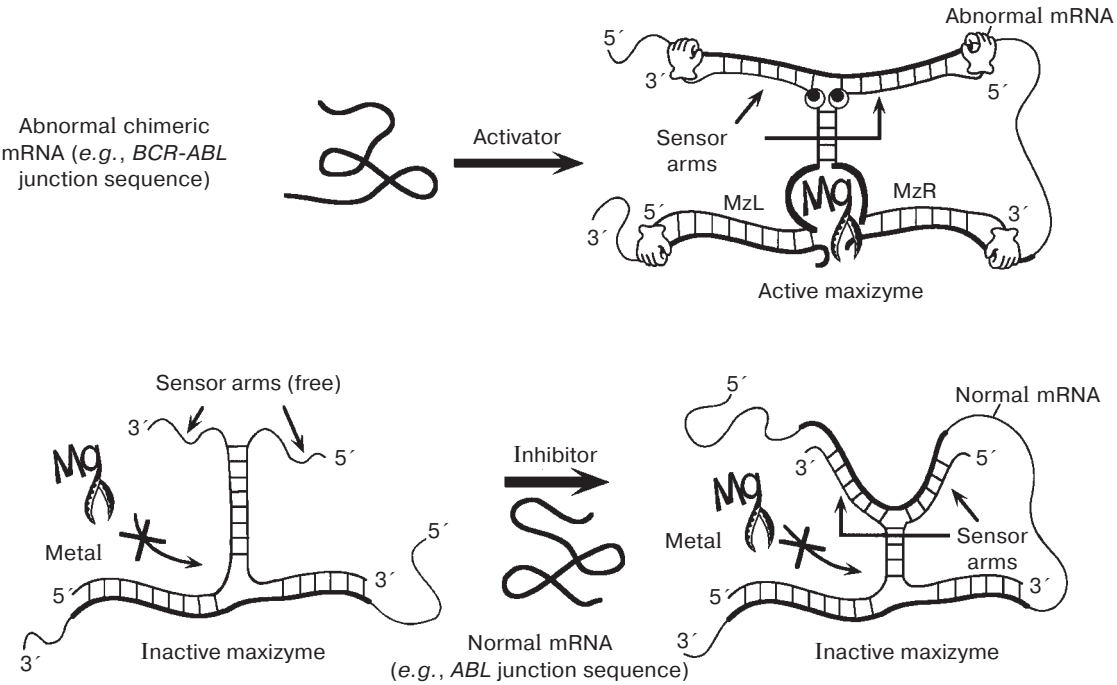

Fig. 2. Development of an allosterically controllable maxizyme. Formation of an active or inactive maxizymes by dimerizations regulated allosterically by specific effector sequences.

inactivation by ribozymes *in vivo* depends strongly on the design of the expression vector. The design can determine both the level of expression and the half-life of the expressed ribozyme. In previous studies, we found that pol III-mediated expression of ribozymes as tRNA fusions resulted in highly expressed stable ribozymes.[15–17]

However, even these improved ribozymes were sometimes ineffective, probably because the ribozyme was unable to locate its target. One potential explanation for this ineffectiveness is that the rate-limiting step *in vivo* for the cleavage of phosphodiester bonds is the annealing/association of the ribozyme with its target site.[17] Furthermore, in a long RNA chain, significant numbers of target sites are not accessible to the ribozyme since they are hidden within secondary or tertiary structures. This problem is often critical in attempts to exploit ribozyme activity, in particular, *in vivo*.

To overcome the problem of accessibility, computer generated secondary structure predictions are typically used to identify targets most likely to have an open conformation. However, these predictions are often inaccurate because of unpredictable RNA—protein interactions that change the structure of RNA in cells. To circumvent this limitation, sometimes an unwieldy systematic approach involving huge numbers of candidate antisense molecules is employed.[14] To avoid being dependent on either of these approaches, we sought to develop a ribozyme that would be able to access any chosen target site independent of local secondary structure.[18]

We reasoned that it would be useful to design a ribozyme that could recruit a protein that could, in turn, relieve any interfering secondary structure, thereby making any site accessible to the ribozyme. To accomplish this, we tried to link a ribozyme to an RNA helicase(s), proteins demonstrated to have nonspecific RNA binding, sliding, and unwinding activities (Fig. 3). We introduced an RNA motif, the RNA helicase-binding motif (RBM) that interacts with RNA helicases *in vitro* and *in vivo*.[18] To investigate whether the protein that binds to the $tRNA^{val}$-driven RBM-attached ribozymes has unwinding and cleavage activities, we performed *in vitro* cleavage assay by these ribozyme-protein complexes. Since RNA helicases possess RNA-unwinding activity, ribozymes with RBM is capable of recruiting this helicase(s) to the target site where it unwinds inhibitory structures. It is attractive to consider that helicase can even be able to slide the tRNA-Rz along a transcript consistent with the sliding mechanisms of action demonstrated for several RNA helicases. The key aspect of our hybrid-rybozyme is that it overcomes a major obstacle of ribozyme use, thereby greatly increasing the general utility of ribozymes. In particular, attachment of the RBM has made it possible to suppress expression of genes that previously were found to be recalcitrant to ribozyme cleavage.[18]

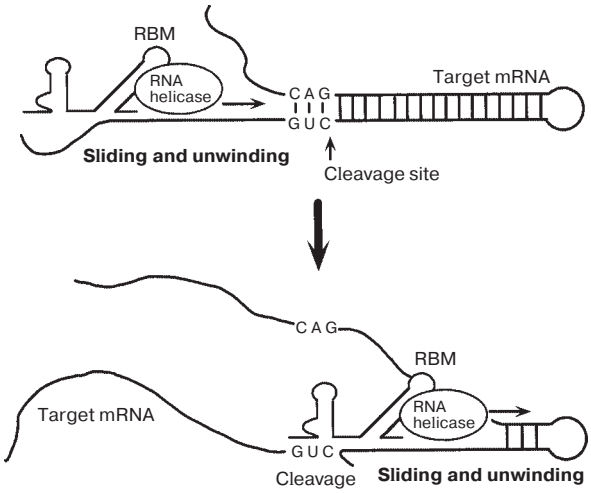

Fig. 3. Schematic representation of how the cleavage of a hidden target site would occur by the hybrid-ribozyme.

At first we generated duplexes as substrates by hybridizing partial mRNAs and mixed with RBM-connected or -unconnected ribozyme-protein complexes. As shown in Fig. 4, RBM-unconnected ribozymes did not unwind the duplexes and, thus, they were unable to cleave the substrate. By contrast, RBM-attached ribozyme-protein complexes were clearly capable of unwinding and cleaving the substrate. However, the inactive ribozyme-protein complex (I-Rz2-RBM, with a single G-to-A mutation at the catalytically important conserved nucleotide) could unwind duplexes but did not cleave the substrate. Thus, these results clearly demonstrate that RBM-attached ribozyme-protein complexes (hybrid-ribozymes) had two activities such as unwinding and cleavage and those unwinding activities were due to the RNA helicase. Thus, importantly, the novel hybrid-ribozymes could cleave inaccessible target sites that were not cleavable by conventional ribozymes.

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. Since our hybrid ribozymes can attack any site, they can attack any mRNA.[18–21] 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.[22–25]

This procedure was used to establish a novel functional gene screening system for the signal pathway of Fas-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 Rz—RBM introduced cells with the Fas-specific antibodies, cells that survived were collected and a respective genomic DNA was isolated from each clone. Sequencing of the randomized region of

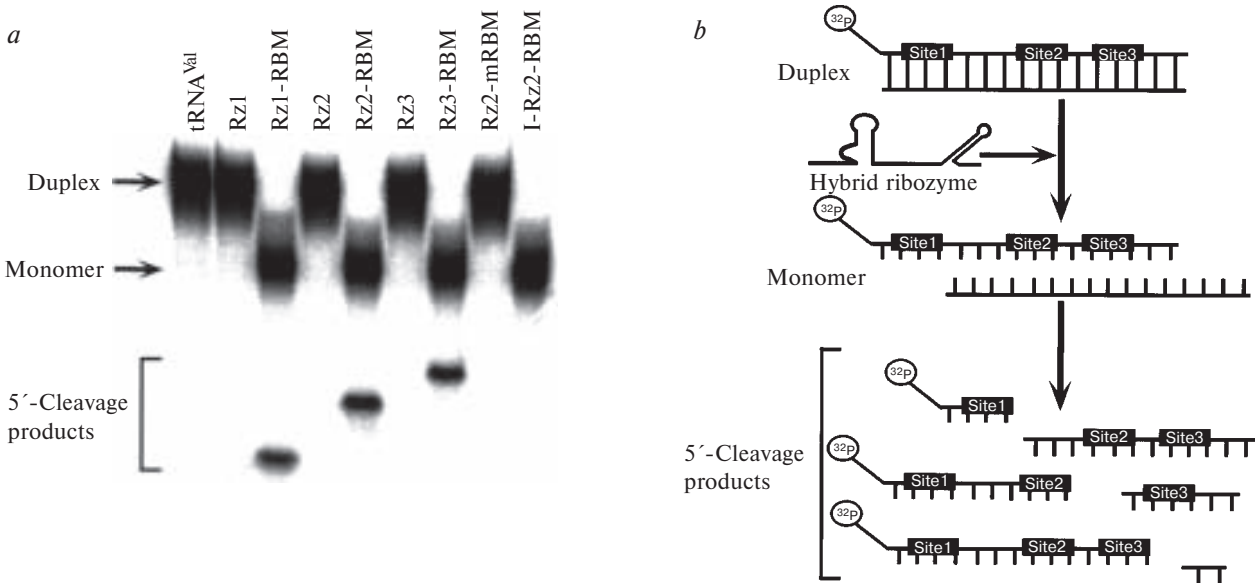

Fig. 4. RBM-connected ribozyme—protein complexes have two activities such as unwinding and cleavage *in vitro*. *a*. Cleavage activity *in vitro* of RBM-connected or -unconnected ribozyme-protein complexes. *b*. Schematic representation of unwinding and cleavage assays.

Rzs—RBM in each genomic DNA and the target genes of ribozymes were identified in databases by a BLAST search. Thus, we can rapidly identify genes that are responsible in the apoptotic pathway. Then we identified many pro-apoptotic genes such as FADD, Caspase 9 and PTEN.[24—26] In addition, we also identified novel genes using this strategy (Fig. 5). It should be emphasized that, in the absence of the RMB, we would not have identified many genes in our screening. Thus, our gene discovery system using hybrid-ribozyme libraries should be useful for the rapid identification of functional genes in the post-genome era.

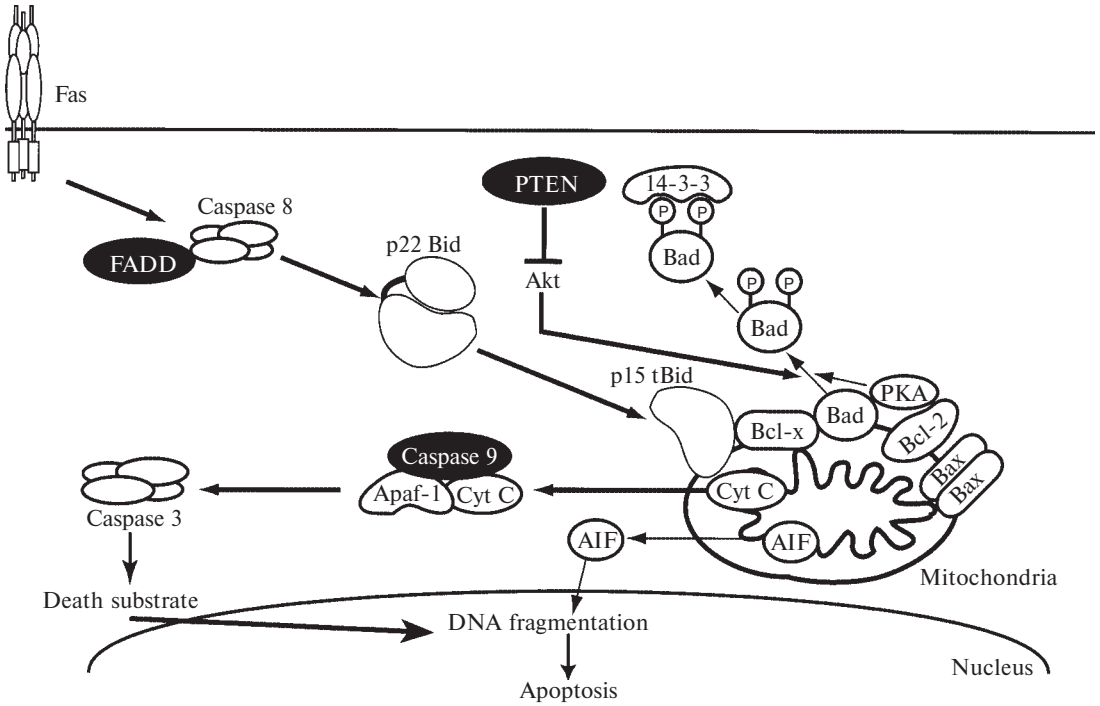

Fig. 5. Identification of functional genes in the Fas-mediated pathway to apoptosis by the gene discovery system. Black boxes indicate identified genes.

Experimental

Construction of vectors that encode RBM-connected ribozymes. The construction of ribozyme-expression vectors derived from plasmid pUC-dt was described previously.[15] To generate RBM-connected Rz-expression vectors, we inserted a RBM sequence. pUC-dt was double-digested with *Csp*45I and *Sal*I. Each individual ribozyme sequence with *Kpn*I and *Eco*RV sites and the terminator sequence UUUUU at the 3′ end were cloned into the plasmid. The *Kpn*I and *Eco*RV sites were used for subsequent insertion of the RBM sequence.

Assays of the activities of RBM-connected or -unconnected ribozyme-protein complexes *in vitro*. RBM-connected or -unconnected ribozyme-protein complexes were described in the text. Partial mRNAs used as substrates were labeled with [γ-^{32}P]-ATP by T4 polynucleotide kinase. Duplexes of substrates used in this assay were prepared by hybridizing the respective ^{32}P-labeled mRNAs as described elsewhere. *In vitro* unwinding and cleavage assays by respective ribozymes were described elsewhere.[4,27]

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