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Therapeutic RNA and DNA Enzymes

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ABSTRACT. Recent progress in understanding how gene products interact in the control of cell proliferation has engendered high hopes for the rational design of specific therapeutic strategies. The demonstration that certain RNA and DNA nucleic acids can enzymatically cleave mRNAs has offered the possibility of inactivating abnormal gene expression. In principle, this technology is applicable to any disease where a specific gene product can be linked to the initiation and/or perpetuation of the disease. Here, a brief description of the technology that can be useful for the design of therapeutic DNA and RNA agents capable of inducing apoptosis in cancer cells is presented. Furthermore, such agents can be a valuable tool for probing gene function. BIOCHEM PHARMACOL **60**;8:1023–1026, 2000. © 2000 Elsevier Science Inc.

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Inhibition of gene expression is an important experimental method to dissect the function of genes and could have a great impact upon the treatment of diseases in which gene products are involved. The identification of short ribonucleotides with endoribonuclease activity has provided researchers with potentially important tools to block the expression of specific genes in cells [1]. The *trans*-acting hammerhead ribozyme, which has been developed by Haseloff and Gerlach [2], is the smallest and perhaps the simplest RNA enzyme with endoribonuclease activity. This ribozyme consists of three helical stems including nine conserved nucleotides that are responsible for the formation of the catalytic domain as illustrated in Fig. 1A. The target site for the ribozyme can have any sequence as long as it contains UH, where H can be any nucleotide except G. Cleavage specificity of the ribozyme is determined by its hybridizing arms, which anneal with the target mRNA in a complementary fashion. Following binding, the ribozyme cleaves its mRNA target. After dissociation, the ribozyme is capable of cleaving more mRNA molecules, thus fulfilling the definition of an enzyme (Fig. 1B). In addition to RNA enzymes, *in vitro* selection experiments have recently been used to select a DNA enzyme capable of cleaving any target RNA at a phosphodiester bond located between an unpaired purine and paired pyrimidine (Fig. 2). The mechanism of cleavage of the DNA enzymes is similar to the RNA enzymes [3]. We have been interested in developing RNA and DNA enzymes that can be used to study gene function and treat diseases in which gene products are involved. RNA enzymes can be delivered to the cells either endogenously as gene-encoding ribozymes [4, 5] or exogenously as preformed ribozymes [6].

Notably, growth factors and cytokines bind specific plasma membrane receptors and activate a complex network of intracellular kinase cascades. Disturbance of such signaling pathways can cause diseases. One of the most important pathways that accounts for the pathology of many diseases is apoptosis [7]. Apoptosis corresponds to a series of changes that cells undergo during programmed or physiological cell death [8]. Failure of cell death can be a major mechanism by which tumours progress to severe stages. Thus, there is considerable interest in evaluating whether specific inhibition of gene products that protect cancer cells from apoptosis will lead to tumour growth inhibition.

DESIGN OF NUCLEASE-RESISTANT RNA AND DNA ENZYMES

Unmodified RNA and DNA are subject to rapid nuclease degradation. In the case of hammerhead ribozyme, the 2′-hydroxyl groups of pyrimidines were found to be the primary sites of ribonucleases [9]. Thus, replacement of natural pyrimidines with their 2′-modified versions should increase ribozyme stability. In this respect, the substitution of pyrimidines with 2′-fluoro, 2′-amino, 2′-*O*-methyl, or 2′-*O*-allyl analogs has produced stable ribozymes [10–12]. Notably, the chemical modifications that maximize ribozyme stability and cleavage activity must be chosen carefully so as not to inhibit ribozyme catalytic activity [11, 12]. Indeed, the substitution of all pyrimidine nucleotides in a ribozyme by their 2′-amino or 2′-fluoro analogs inhibited ribozyme cleavage activity by 25- to 50-fold compared to the unmodified ribozyme [11]. In contrast to these studies, we have found that the replacement of all

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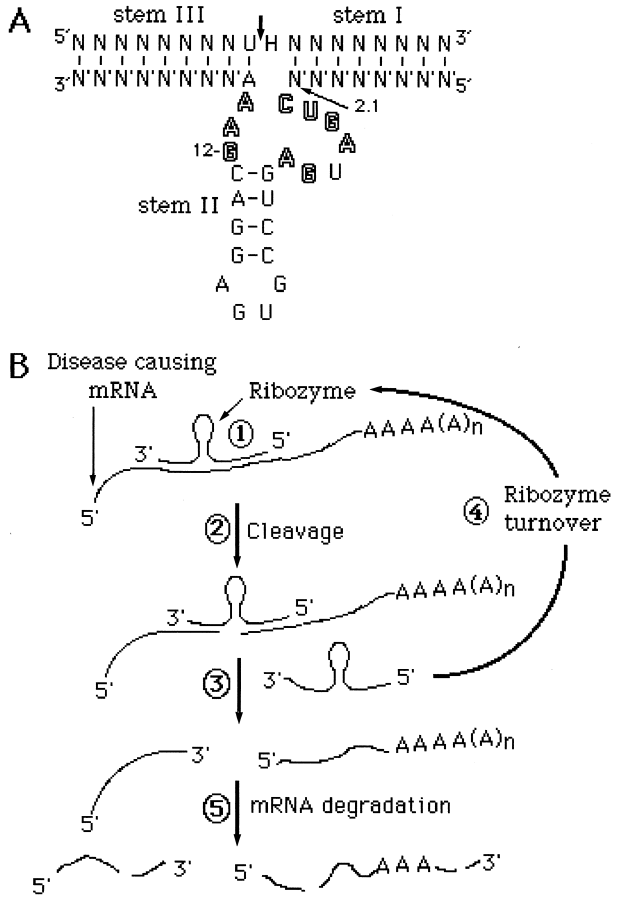

FIG. 1. The hammerhead ribozyme and its catalytic cycle. (A) Structure of the ribozyme/RNA target complex. Outlined letters indicate the conserved nucleotides. The cleavage site is indicated by an arrow. (B) Catalytic cycle of the ribozyme. This cycle consists of (1) sequence-specific binding of the ribozyme to a disease causing mRNA. After cleavage (2), the ribozyme is dissociated from the mRNA (3) and then cleaves a second mRNA molecule (4). The cleaved mRNA is subjected to rapid degradation by cellular ribonucleases (5).

pyrimidine nucleotides by their 2′-amino analogs can produce stable RNA enzymes with high stability and cleavage activity, provided that position 2.1 contains a purine or an unmodified pyrimidine [13–15]. The presence of 2′-amino at position 2.1 may influence ribozyme/substrate structure and therefore cleavage efficacy [15]. This observation should facilitate the design of totally 2′-amino pyrimidine-modified ribozymes for exogenous delivery in different therapeutic settings.

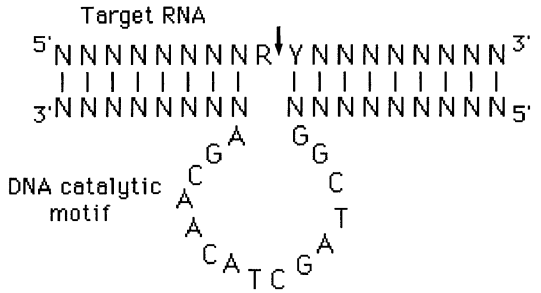

FIG. 2. The 10–23 DNA catalytic motif selected by Santoro and Joyce [3]. The arrow indicates the cleavage site.

In addition to developing RNA enzymes, we have also explored the possibility of selecting DNA molecules with endoribonuclease activity from random deoxynucleotide libraries. Some of the selected motifs showed a poor cleavage activity.* In contrast to this, Santoro and Joyce recently selected very active DNA enzymes [3], in particular the 10–23 motif (Fig. 2). Due to its DNA form, its high cleavage activity at physiological Mg^{2+} concentration, and its easy synthesis and manufacturing, the 10–23 motif may represent the optimal nucleic acid drug to date. When tested for its stability in serum, however, it was found to be rapidly degraded by serum nucleases. By introducing either a 3′-3′-inverted thymidine nucleotide or phosphorothioate analogs in the antisense arms and in the pyrimidine residues of the catalytic core, we have designed stable DNA enzymes that retained significant cleavage activity and cell efficacy [16].

SIGNALING PATHWAY-TARGETED THERAPY

Notably, apoptotic signals are activated by different stimuli and converge toward a common death pathway, for which Bcl-2† family proteins function as regulators and caspase family proteases function as signal transducers [7]. The gene expression and activity of the Bcl-2-related protein can be regulated by various kinases and phosphatases. Defining the kinases that regulate the gene expression and activity of the Bcl-2-related proteins may open new therapeutic strategies.

We began by investigating whether there is a direct link between the PKC signal pathway and apoptosis. PKC is a family of serine–threonine protein kinases which contains at least 12 isoforms. Based upon their biochemical properties and sequence homologies, they have been divided into three subclasses [17, 18]. Although PKC activity has been shown to be increased in some tumours, the precise roles of each isoform in sustained tumour growth are essentially unknown. Therefore, we have analyzed the expression of PKC isoforms and Bcl-2-related proteins in some cancer cell lines, such as gliomas. Most cell lines were found to up-regulate the expression of PKCα and Bcl-x_L, suggesting a possible molecular interaction between these two genes. Both PKCα and δ were found to be associated with the membrane fraction, suggesting their constitutive activation in glioma cells [13]. This constitutive activation may be responsible for the sustained tumour cell growth.

To see whether the up-regulation of PKCα in glioma cells is important for malignant glioma cell proliferation, its expression was targeted by a nuclease-resistant RNA enzyme in which all the 2′-hydroxyl pyrimidines were substi-

* Sioud M, unpublished results.

† *Abbreviations:* Bcl, B-cell lymphoma; PKC, protein kinase C; Apaf-1, apoptotic protease-activating factor-1; MDR, multidrug resistance; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*, *N*, *N*-trimethylammonium-methyl sulfate; and Rz, ribozyme.

tuted with their 2′-amino analogs. The ribozyme inhibited glioma cell proliferation *in vitro* and *in vivo* [13]. In PKCα ribozyme-treated cells, there was a significant reduction in both the PKCα and Bcl-x_L protein levels [13, 19]. Further analysis has shown that both proteins are localized within the mitochondrial membrane [16]. The mitochondrial membrane-associated PKCα may directly regulate the function of Bcl-x_L through phosphorylation. In accordance with our observation, Ruvolo *et al.* [20] recently demonstrated that the overexpression of the PKCα in REH cells increased mitochondrial PKCα localization, augmented Bcl-2 phosphorylation, and enhanced resistance to apoptosis induced by anticancer drugs. Both Bcl-2 and Bcl-x_L proteins are predominantly localized in the outer mitochondrial membrane, where they inhibit the association of the Apaf-1 with procaspase-9 and thereby prevent the initiation of apoptosis [21]. Based upon the data described above, it would appear that the PKCα isoform is the kinase for Bcl-2 and Bcl-x_L proteins. Notably, the overexpression of PKCα was also found to be associated with the multidrug-resistant phenotype [22]. This phenotype is closely associated with a decrease in intracellular drug accumulation and the overexpression of a 170-kDa glycoprotein, P-glycoprotein [23].

An alternative approach to RNA enzymes is the use of DNA enzymes [4]. In connection with this, we have developed nuclease-resistant PKCα enzymes that induced apoptosis in sensitive cancer cell lines [16]. In contrast to the PKCα RNA ribozyme, the PKCα DNA enzyme was found to be mainly localized within the nucleus. This nuclear localization is expected to increase the rate of ribozyme binding to mRNA during transcription and hence increase its effectiveness.

ANGIOGENESIS-TARGETED THERAPY

One potential target pathway for the treatment of solid tumours is angiogenesis. Indeed, the formation of the vascular network is essential for solid tumour to grow and metastasize [24]. Although a large number of angiogenic factors produced by tumour cells themselves and by accessory host cells have been implicated in promoting tumour angiogenesis, both fibroblast and vascular endothelial growth factors (FGF and VEGF) were considered as the most potent angiogenic factors [25, 26]. Malignant gliomas are rapidly dividing cells with a high degree of vascularity. To interfere with VEGF expression, we have developed a nuclease-resistant VEGF RNA enzyme [14]. Figure 3A shows the ribozyme base-paired with its mRNA target site. Interestingly, a single injection of 200 μg into a glioma solid tumour inhibited subcutaneous tumour growth in syngenic rats. Figure 3B shows hematoxylin/eosin-stained frozen 5 μm cryostat sections from DOTAP-treated tumour (control) and DOTAP/VEGF ribozyme-treated tumour. Notably, tumour treated with the liposome (DOTAP)-formulated VEGF ribozyme showed a very significant reduction in glioma cell number. The sections were per-

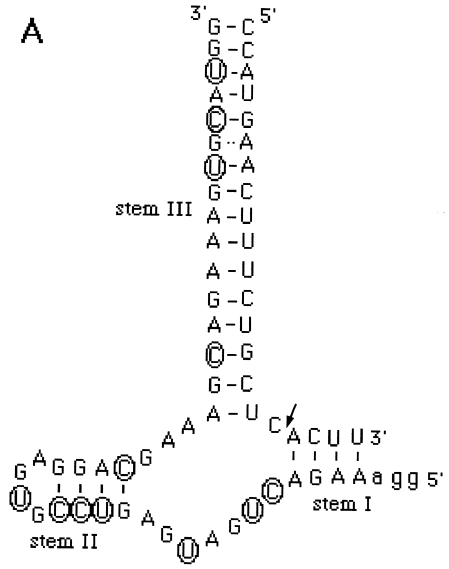

FIG. 3. Sequence and *in vivo* activity of the VEGF ribozyme. (A) Base pairing of the ribozyme with its target mRNA. The 2′-amino pyrimidine nucleotides in the modified ribozyme are circled. The cleavage site is indicated by an arrow. (B) Hematoxylin/eosin-stained frozen 5-μm cryostat sections from a DOTAP-treated glioma tumour and a VEGF ribozyme (Rz)-treated glioma tumour.

formed at 20 days following ribozyme treatment. Interestingly, ribozymes directed against the VEGF receptor mRNAs (Flt-1 and KDR) were found to reduce neovascularization in a rat corneal model of VEGF-induced angiogenesis [27]. Taken together, the data indicate a crucial role for VEGF in angiogenesis.

CONCLUSION

The feasibility of cleaving mRNA selectively by RNA and DNA enzymes should hold a great promise for clinical medicine. Furthermore, these agents can be valuable tools for the delineation of gene function. Such biological information is important for the design of rational new therapeutic strategies based on knowledge.

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References

1. Uhlenbeck OC, A small catalytic oligonucleotide. *Nature* **328:** 596–619, 1987.
2. Haseloff J and Gerlach WL, Simple RNA enzymes with new and highly specific endoribonuclease activities. *Nature* **334:** 585–591, 1988.
3. Santoro SW and Joyce GF, A general purpose RNA-cleaving DNA enzyme. *Proc Natl Acad Sci USA* **94:** 4262–4266, 1996.
4. Cotten M and Birnstiel ML, Ribozyme mediated destruction of RNA *in vivo*. *EMBO J* **8:** 3861–3866, 1989.
5. Sioud M and Drlica K, Prevention of HIV-1 integrase expression in *E. coli* by a ribozyme. *Proc Natl Acad Sci USA* **88:** 7303–7307, 1991.
6. Sioud M, Natvig JB and Førre Ø, Preformed ribozyme destroys tumour necrosis factor mRNA in human cells. *J Mol Biol* **223:** 7303–7307, 1992.
7. Kroemer G, The proto-oncogene Bcl-2 and its role in regulating apoptosis. *Nat Med* **3:** 614–620, 1997.
8. Jacobson MD, Weil M and Raff MC, Programmed cell death in animal development. *Cell* **88:** 347–354, 1997.
9. Heidenreich O, Pieken W and Eckstein F, Chemically modified RNA. *FASEB J* **7:** 90–96, 1993.
10. Pieken WA, Olsen DB, Benseler F, Aurup H and Eckstein F, Kinetic characterization of ribonuclease-resistant 2′-modified hammerhead ribozymes. *Science* **253:** 314–317, 1991.
11. Heidenreich O, Benseler F, Fahrenholz A and Eckstein F, High activity and stability of hammerhead ribozymes containing 2′-modified pyrimidine nucleotides and phosphorothioates. *J Biol Chem* **269:** 2131–2138, 1994.
12. Beigelman L, McSwiggen JA, Draper KG, Gonzalez C, Jensen K, Karpeisky AM, Modak AS, Matulic-Adamic J, Direnzo AB, Haeberli P, Sweedler D, Tracz D, Grimm S, Wincott FE, Thackray VG and Usman N, Chemical modification of hammerhead ribozymes. Catalytic activity and nuclease resistance. *J Biol Chem* **270:** 25702–25708, 1995.
13. Sioud M and Sørensen R, A nuclease-resistant protein kinase Cα ribozyme blocks glioma cell growth. *Nat Biotech* **16:** 556–561, 1998.
14. Leirdal M and Sioud M, High cleavage activity and stability of hammerhead ribozymes with a uniform 2′-amino pyrimidine modification. *Biochem Biophys Res Commun* **250:** 171–174, 1998.
15. Sioud M and Leirdal M, Substitution of the 2′-hydroxyl group at position 2.1 by an amino group interferes with Mg^{2+} binding and efficient cleavage by hammerhead ribozymes. *Biochem Biophys Res Commun* **262:** 461–466, 1999.
16. Sioud M and Leirdal M, Design of nuclease resistant protein kinase C α DNA enzymes with potential therapeutic application. *J Mol Biol* **296:** 937–947, 2000.
17. Newton AC, Protein kinase C: Structure, function and regulation. *J Biol Chem* **270:** 28495–28498, 1995.
18. Parker PJ, Kour G, Marais R, Mitchell F, Pears C, Stabel S and Webster C, Protein kinase C—α family affair. *Mol Cell Endocrinol* **65:** 1–11, 1989.
19. Leirdal M and Sioud M, Ribozyme inhibition of the protein kinase Cα triggers apoptosis in glioma cells. *Br J Cancer* **80:** 1558–1564, 1999.
20. Ruvolo PP, Deng X, Carr BK and May WS, A functional role for mitochondrial protein kinase Cα in Bcl2 phosphorylation and suppression of apoptosis. *J Biol Chem* **273:** 25436–25442, 1998.
21. Hu Y, Benedict MA, Wu D, Inohara N and Nunez G, Bcl-x_L interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. *Proc Natl Acad Sci USA* **95:** 4386–4391, 1998.
22. Kerr JF, Wyllie AH and Currie AR, Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26:** 239–257, 1972.
23. Kartner N, Riordan JR and Ling V, Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. *Science* **221:** 1285–1287, 1983.
24. Folkman J, Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* **1:** 27–31, 1995.
25. Basilico C and Moscatelli D, The FGF family of growth factors and oncogenes. *Adv Cancer Res* **59:** 115–165, 1992.
26. Zetter BR, Angiogenesis and tumor metastasis. *Annu Rev Med* **49:** 407–424, 1998.
27. Parry TJ, Cushman C, Gallegos AM, Agrawal AB, Richardson M, Andrews LE, Maloney L, Mokler VR, Wincott FE and Pavco PA, Bioactivity of anti-angiogenic ribozymes targeting Flt-1 and KDR mRNA. *Nucleic Acids Res* **27:** 2569–2777, 1999.