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High Cleavage Activity and Stability of Hammerhead Ribozymes with a Uniform 2′-Amino Pyrimidine Modification

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The uniform 2′-pyrimidine modifications have been found to inhibit ribozyme cleavage activity. However, in the present study we show that a good cleavage activity can be achieved for the 2′-amino modified ribozymes when their sequences were designed to contain only a few pyrimidines in helix I. In particular, ribozymes with no pyrimidines in helix I cleaved their target RNAs with almost the same efficacy as their unmodified versions. Interestingly, selective 2′-amino modification at positions 2.1 and 2.2 reduced the ribozyme cleavage activity by 8-fold, suggesting that the 2′-amino groups at these two positions may interfere with the formation of the ribozyme active conformation. In addition, uniformly modified ribozymes showed a remarkable stability in serum. Taken together these results should facilitate the design of stable ribozymes with sustained cleavage activity.

Recent experiments indicate that hammerhead ribozymes can be designed to cleave *in trans* virtually any RNA whose sequences are known (1-8). However, the therapeutic utility of exogenously delivered ribozymes is potentially limited by their short half-life in biological fluids. In order to improve stability many chemical modifications have been introduced into ribozymes (9-14). Introduction of 2′-amino uridines at position U4 and U7 in a ribozyme with 2′-fluoro pyrimidines increased the ribozyme stability without affecting its cleavage activity (11). Furthermore, ribozymes with 2′-*0*-methyl nucleotides having one or two 2′-modified riboses at position U4 and/or U7 retained their cleavage activity when compared to their unmodified versions (12). In contrast to these selective chemical modifications, uniform modifications in hammerhead ribozymes were found to reduce significantly their cleavage activity. For example, replacement of all pyrimidine nucleotides with their 2′-fluoro or 2′-amino analogs resulted in 20 to 50-fold decrease in ribozyme cleavage activity, respectively (9), while substitution of the 2′-hydroxyl groups by 2′-*O*-methyl-2′-deoxyuridine/cytidine inactivated the ribozyme (14). These studies suggested that the strategy of uniform modification cannot be directly applied to hammerhead ribozymes, since the cleavage activity of the modified ribozymes was nearly abolished.

We have been looking at the effect of pyrimidine content in helix I and III upon the cleavage activity of ribozymes with uniform 2′-modifications. In this study, we show that hammerhead ribozymes with low pyrimidine content, especially in helix I, can be fully substituted with 2′-amino pyrimidines without detriment to the catalytic activity.

MATERIALS AND METHODS

In vitro RNA synthesis. Unmodified and 2′-amino pyrimidine modified ribozymes were synthesised by *in vitro* transcription using DNA oligodeoxy-nucleotides and the T7 RNA polymerase as previously described (4,5). Following transcription, intact ribozymes were gel-purified, eluted, ethanol-precipitated, washed with 70% ethanol, dried and resuspended in water and their concentration was determined by measurement of absorbency at 260 nm. The 2′-amino modified nucleotides were obtained from Amersham (Little Chalfont, United Kingdom) and used as substrates for the T7 RNA polymerase as described by Aurup et al. (15). Target RNAs corresponding to the vascular endothelial growth factor (VEGF) ribozyme cleavage site was synthesized by *in vitro* transcription of a synthetic DNA template with the T7 RNA polymerase and subsequently gel-purified. After transcription, the gel-purified substrate RNA was dephosphorylated by alkaline phosphate and then 5′-end labelled using T4 polynucleotide kinase and [γ-^{32}P] ATP. The RNA substrates for the mouse tumour necrosis factor α (TNFα) was chemically synthesised. The unmodified, modified TNFα ribozyme with 2′-amino uridine at position 2.1 and 2.2 and the RNA substrate were chemically synthesised by Dr. Phil Hendry and Maxine McCall (CSIRO, Sydney, Australia).

In vitro cleavage activity of the unmodified and modified ribozymes. Cleavage reactions were performed at 37°C in buffer containing 50

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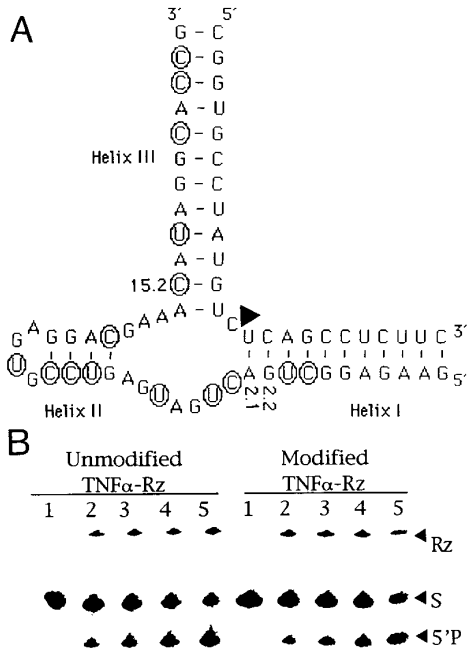

FIG. 1. *In vitro* cleavage activity of the TNFα ribozyme. (A) Base-pairing of the TNFα ribozyme with its RNA target site. The cleavage site is indicated by an arrow. The 2′-amino pyrimidine nucleotides in the modified ribozyme are circled. (B) An example of multiple turnover reaction kinetics of the unmodified and 2′-amino pyrimidine modified TNFα ribozyme. Equal amounts of *in vitro* transcribed and internally labelled ribozymes (10 nM) were incubated with 100 nM of 5′-^{32}P-end-labelled target RNA in reaction mixtures containing 50 mM Tris-HCl, pH 7. 4, and 10 mM $MgCl_2$ at 37°C. Aliquots were taken at the time points 10, 20, 30 and 60 min (lanes 2-5, respectively), analysed by electrophoresis on a 15% polyacrylamide with 7 M urea and visualised by PhosphoImager. The 5′-cleavage product is indicated by an arrow. Rz, ribozyme; S, substrate; 5′P, 5′-cleavage product.

mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$. Cleavage products were separated by electrophoresis on a 15% polyacrylamide gel containing 7 M urea and scanned on a Molecular Dynamics PhosphoImager. Initial ribozyme cleavage rates and k_{cat} were determined from Lineweaver-Burk plots.

Ribozyme stability analysis in fetal calf serum (FCS). [α-^{32}P] ATP internally labelled ribozymes were incubated in medium containing 10% FCS. Aliquots of the mixture were removed at various times, quenched with phenol/chloroform mixture and frozen until use. All samples were phenol extracted and analysed on 15% polyacrylamide gel with 7 M urea and scanned by a Molecular Dynamics PhosphoImager.

RESULTS

In Trans Cleavage Activity of Ribozymes with a Complete 2′-Amino Pyrimidine Modification

Recent experiments have shown loss in cleavage activity of totally 2′-amino pyrimidine substituted ribozymes (9,13). In particular, where all pyrimidines had been replaced by their 2′-amino analogs only 1.9% cleavage activity was retained (9). To our surprise, when the cleavage activity of a 2′-amino pyrimidine modified rat PKCα ribozyme was investigated, it was found to be 40 to 60% of the unmodified ribozyme activity. Indeed, the unmodified and the modified PKCα ribozyme cleaved the short RNA substrate with an apparent turnover number of 0.32 min^{-1} and 0.20 min^{-1}, respectively. Most importantly, the modified ribozyme blocked tumour growth *in vivo* (16).

To see whether this similarity in cleavage also applied to other ribozymes, all pyrimidine nucleotides in a TNFα ribozyme were substituted with their 2′-amino analogues (Fig. 1A). Both ribozymes cleaved the RNA substrate with comparable efficacy (Fig. 1B). The high cleavage activity of the modified TNFα ribozyme is in disagreement with the data reported by Pieken et al. where a chemically and uniformly modified 2′-amino

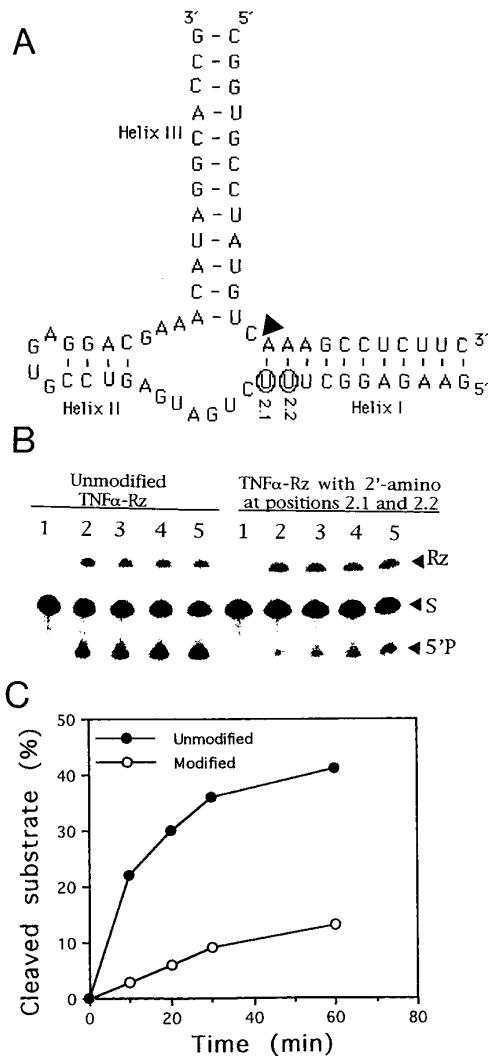

FIG. 2. *In vitro* cleavage activity of the TNFα with 2′-aminouridine at position 2.1 and 2.2. (A) Base-pairing of the ribozyme with its RNA target site. The cleavage site is indicated by an arrow. The 2′-amino uridines are circled. (B) An example of multiple turnover reactions of unmodified and modified ribozymes. Cleavage reactions are as in Figure 1B. (C) Quantification of the data shown in B.

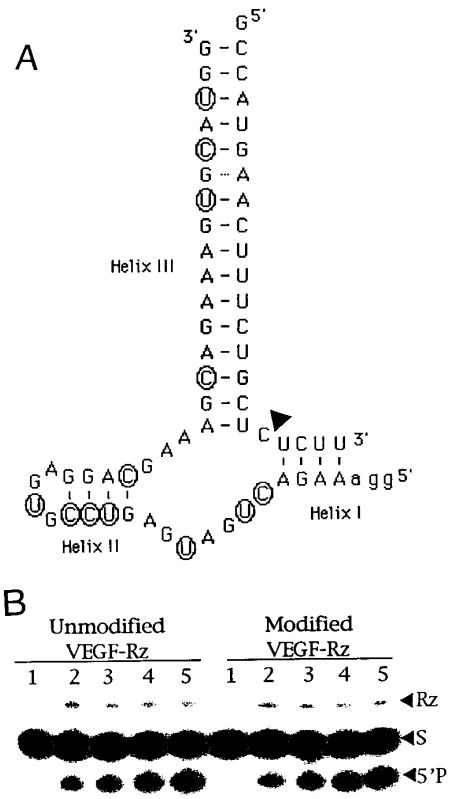

FIG. 3. *In vitro* cleavage activity of the VEGF ribozyme. (A) Base-pairing of the VEGF ribozyme with its RNA target site. The cleavage site is indicated by an arrow. The 2′-amino pyrimidines are circled. (B) An example of multiple turnover reactions of unmodified and modified VEGF ribozymes. PhosphorImager printout of a 15% denaturing polyacrylamide gel. Cleavage reactions were carried out at 37°C with 5 nM ribozyme and 100 nM substrate in the presence of 50 mM Tris-HCl, pH 7.4, and 10 mM $MgCl_2$. Aliquots were taken at the time points 5, 10, 15 and 30 min (lanes 2-5, respectively), analysed by electrophoresis on a 15% polyacrylamide with 7 M urea and visualised by PhosphoImager.

pyrimidine ribozyme showed almost no cleavage activity (9). As hammerhead ribozymes have virtually the same catalytic core, the inhibition of cleavage in their case must be due to the presence of 2′-amino pyrimidines in helix I and/or III.

The Presence of 2′-Amino Groups at Positions 2.1 and 2.2 Inhibits the Ribozyme Cleavage Activity

To evaluate the effect of the 2′-amino groups on the ribozyme cleavage activity as a first step, we investigated the effect of a 2′-amino group at position 2.1 (17). A TNFα ribozyme identical to the one shown in figure 1, but with a cytidine (C) at position 2.1 and a guanidine (G) in the corresponding position in the substrate was designed. Complete 2′-amino pyrimidine modification reduced the ribozyme cleavage activity by approximately 6-fold. This data would suggest a negative effect of the 2′-amino group at position 2.1 on the ribozyme catalytic potency. Complete 2′–amino pyrimidine substitution in a second TNFα ribozyme containing pyrimidines at positions 2.1 and 2.2 reduced the ribozyme cleavage activity by almost 7 to 8-fold.

To gain further insight into the effect of the 2′- amino groups at positions 2.1 and 2.2 on ribozyme cleavage activity we have performed a selective modification. A TNFα ribozyme identical to the one shown in figure 1, but with 2-amino uridines at only positions 2.1 and 2.2 was chemically synthesised (Fig. 2A). Interestingly, such specific modification reduced the ribozyme cleavage activity by almost 8-fold (Fig. 2B and C, as a representative example).

Design of Ribozymes That Can Be Totally 2′-Amino Pyrimidine Substituted

Although the catalytic potency of hammerhead ribozymes might be in part influenced by their secondary structures following 2′-pyrimidine modifications, analysis of several ribozymes indicated that ribozymes containing purines at position 2.1, 2.2 and 15.2 have their catalytic activities either unaffected or slightly affected by the 2′-amino pyrimidine modifications. To illustrate this observation, ribozyme directed against VEGF was designed and its *in vitro* cleavage activity was investigated. In addition to many biological roles, VEGF plays a crucial factor in tumour angiogenesis and metastasis (18). Figure 3A shows the VEGF ribozyme complexed with its corresponding cleavage site within the rat VEGF mRNA sequence. As can be seen, the ribozyme was designed to contain no pyrimidines in helix I, while position 15.2 contains a purine (G). The 2′-amino pyrimidine modified ribozyme cleaved the target RNA

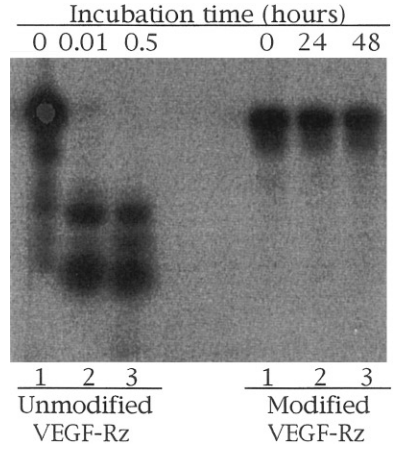

FIG. 4. Ribozyme stability in medium containing 10% FCS. Internally labelled and PAGE purified ribozymes were incubated in RPMI supplemented with 10% FCS. At indicated times 10 μl aliquots were removed from the mixture and processed as described in material and methods. Samples were analysed by 15% polyacrylamide gel with 7 M urea and analysed by PhosphoImager. Time point 0 was taken prior to serum addition.

with almost the same efficacy as the unmodified ribozyme (Fig. 3B). The apparent turnover k_{cat} for the unmodified and modified ribozymes were found to be 1.4 ($\pm$ 0.15) min^{-1} and 1.32 ($\pm$ 0.12) min^{-1}, respectively.

In Vitro Stability of the Unmodified and Modified Ribozymes

One of the major problems associated with exogenous delivery of ribozymes is their sensitivity to nucleases present in biological fluids. In this respect, pyrimidines in hammerhead ribozymes have been shown to be a major site for nucleases (19). Thus, we have investigated the effect of the 2′-amino pyrimidine modification on the VEGF ribozyme stability. Internally labelled unmodified or modified ribozyme were incubated in cell culture medium containing 10% FCS. In contrast to the unmodified ribozyme ($t_{1/2}$= 0.1 min) the ribozyme with all 2′-amino pyrimidine nucleotides was found to be stable in 10% FCS. No significant degradation was observed following 48 hours incubation time (Fig. 4). Similar stability results were obtained with the other ribozymes.

DISCUSSION

Our data show that total replacement of all pyrimidines with their 2′-amino analogs can produce ribozymes with sustained cleavage activity. In a previous study, a ribozyme containing 2′-amino pyrimidine groups at all positions was found to be 50-fold less active than its unmodified version (9). Notably, in this ribozyme most positions in helix I and III, especially positions 2.1, 2.2 and 15.2, contain pyrimidines. Therefore during ribozyme design, some attention should be paid to the base composition of helix I. In this respect, if the pyrimidine content of the substrate recognition arms is limited and, in particular, positions 2.1, 2.2 and 15.2 are purines then the ribozymes can be totally substituted with 2′-amino pyrimidines without loss of the cleavage activity. This observation should facilitate the design of stable and active ribozymes for *in vivo* therapeutic application.

In addition to the demonstration that complete 2′-amino pyrimidine replacement is feasible, the present data also suggest a potentially important role for the 2′-pyrimidine hydroxyl groups at position 2.1 and 2.2 on ribozyme cleavage activity. We are now seeking to understand the molecular basis by which 2′-amino groups, in particular, at position 2.1 inhibit ribozyme cleavage activity.

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REFERENCES

1. Cameron, F. H., and Jennings, P. A. (1989) *Proc. Natl. Acad. Sci. USA* **83**, 8859–8862.
2. Cotten, M., and Birnstiel, M. L. (1989) *EMBO J.* **8,** 3861–3866.
3. Sarver, N., Cantin, E. M., Chan, P. S., Zaia, J. A., Ladne, P. A., Stephens, D. A., and Rossi, J. J. (1990) *Science* **247,** 1222–1225.
4. Sioud, M., and Drlica, K. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7303–7307.
5. Sioud, M. (1994) *J. Mol. Biol.* **242,** 619–629.
6. Scanlon, K. J., Ishida, H., and Kashani-Sabet, M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11123–11127.
7. Heidenreich, O., and Eckstein, F. (1992) *J. Biol. Chem.* **267,** 1904–1909.
8. Birikh, K. R., Heaton, P. A., and Eckstein, F. (1997) *Eur. J. Biochem.* **245,** 1–16.
9. Pieken, W. A., Olsen, D. B., Benseler, F., Aurup, H., and Eckstein, F. (1991) *Science* **253,** 314–317.
10. Williams, D. M., Pieken, W. A., and Eckstein, F. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 918–921.
11. Heidenreich, O., Benseler, F., Fahrenholz, A., and Eckstein, F. (1994) *J. Biol. Chem.* **269,** 2131–2138.
12. Beigelman, L., McSwiggen, J. A., Draper, K. G., Gonzalez, C., Jensen, K., Karpeisky, A. M., Modak, A. S., Matulic-Adamic, J., DiRenzo, A. B., Haeberli, P., Sweedler, D., Tracz, D., Grimm, S., Wincott, F. E., Thackray, V. G., and Usman, N. (1995) *J. Biol. Chem.* **270,** 25702–25708.
13. Burgin, A. B. Jr., Gonzalez, C., Matulic-Adamic, J., Karpeisky, A. M., Usman, N., McSwiggen, J. A., and Beigelman, L. (1996) *Biochemistry* **35,** 14090–14097.
14. Scherr, M., Grez, M., Ganser, A., and Engels, J. W. (1997) *J. Biol. Chem.* **272,** 14304–14313.
15. Aurup, H., Williams, D. M., and Eckstein, F. (1992) *Biochemistry* **31,** 9636–9641.
16. Sioud, M., and Sørensen, R. (1998) *Nature Biotech.* **16,** 556–561.
17. Hertel, K. J., Pardi, A., Uhlenbeck, O. C., Koizumi, M., Ohtsuka, E., Uesugi, S., Cerdergren, R., Eckstein, F., Gerlach, W. L., Hodgson, R., and Symons, R. H. (1992) *Nucleic Acids Res.* **21,** 2809–2814.
18. Zetter, B. R. (1998) *Annu. Rev. Med.* **49,** 407–424.
19. Heidenreich, O., Pieken, W., and Eckstein, F. (1993) *FASEB J.* **7,** 90–96.