In vivo decay kinetic parameters of hammerhead ribozymes

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ABSTRACT

Ribozymes offer a potentially important way to inactivate intracellular RNA from almost any gene whose nucleotide sequence is known. Recently, we found that hammerhead ribozymes directed against mRNA of tumour necrosis factor alpha (TNF α) and its derivatives, preferentially bind to a cellular protein(s). To better understand the effect of different 3'-terminal hairpins on ribozyme stability as well as their effect on the protein binding to the ribozyme, a mathematical treatment of the decay of three TNF α ribozymes that differed at their 3' ends was performed. One ribozyme contained a 3'-terminal hairpin derived from a transcription terminator of bacteriophage T7, another contained the same hairpin but modified to be highly enriched for G+C nucleotides, and a third lacked a hairpin. The TNF α ribozyme decay had two kinetic components. The slow component exhibited exponential decay with a half life of approximately 250 h in all cases. The 3'-terminal hairpin has no significant effect on this component. This slow phase accounted for 60–80% of ribozyme decay. The rapid phase also exhibited exponential decay. For this phase, a 3'-terminal hairpin roughly doubled the half-life (1.7–3.4). The slow phase of degradation was about three times faster for a ribozyme directed at the integrase mRNA of human immunodeficiency virus-1 than that seen with the TNF α ribozyme. Taken together, these results suggest that the ribozyme population is initially sensitive to degradation, with the presence of a hairpin provides some protection, and indicate that the addition of the hairpin to the ribozyme did not prevent the *in vivo* additional stabilizing effect of the protein(s).

INTRODUCTION

The discovery of RNA molecules (ribozymes) that possess enzymatic RNA cleaving activity has led to a new way to artificially control gene expression (1,2). Several types of

ribozyme have been described (3–7). In each case, the ribozyme recognizes its RNA target through complementary base pairing and cleaves the target at a specific site. This cleavage then makes the target accessible to degradation by cellular nucleases. In the few cases examined experimentally, *trans*-acting hammerhead ribozymes are more effective at blocking gene expression than comparable antisense RNAs (8–10) and they seem to have few deleterious effects on cell growth (8,11). Thus there has been considerable interest in developing ribozymes to combat human diseases (9,11–17).

We have been interested in refining hammerhead ribozymes for manipulating gene expression in both prokaryotic (8) and eukaryotic cells (10). Hammerhead ribozymes contain a 22-nucleotide catalytic domain flanked by two short regions that form complementary base pairs with the target (3). Among the factors expected to be important for ribozyme efficacy is intracellular stability. We had previously reasoned that a short palindromic region at the 3' end of the ribozyme would generate a terminal hairpin that would protect the ribozyme from 3' exonuclease digestion (8). When tested in human cells, a 3'-terminal hairpin, in the form of a bacteriophage transcription terminator, did retard ribozyme decay when appended to a ribozyme directed at mRNA from the TNF α gene. In addition, cell fractionation studies demonstrated that a high proportion of the incoming ribozymes is recovered with the nuclei following cell fractionation (10). The slow decay of TNF α ribozyme in both HL60 and PBMN cells has been explained by the recent finding that endogenous cellular protein(s) is providing protection to the ribozyme *in vivo* (19).

To gain further insight into the stabilizing effect of different 3'-terminal hairpins, and their effect on the protein(s) binding to the ribozyme, in the present study we have determined the *in vivo* kinetic parameters of several TNF α ribozymes.

MATERIALS AND METHODS

Preparation of ribozyme RNA

RNA was prepared as described previously (8,10). Two overlapping oligonucleotides were synthesized that contained the

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sequences of 1) a bacteriophage T7 RNA polymerase promoter, 2) the 5' and 3' recognition sequences of the ribozyme (nucleotides complementary to #374–393 of TNF α (18) or nucleotides 433–453 for HIV-1 (BH10) integrase (8), 3) the catalytic domain of the tobacco ring spot virus satellite RNA hammerhead ribozyme (3), 4) in some cases a bacteriophage T7 transcription terminator (20) and 5) *SalI* and *EcoRI* sites at the 5' and 3' ends, respectively. The overlapping oligonucleotides were hybridized and extended with the Klenow fragment of DNA polymerase. The filled DNA oligos were extracted with phenol, precipitated with ethanol, purified by preparative polyacrylamide gel electrophoresis, and cloned into a *SalI*–*EcoRI*-cleaved pUC18 vector. The nucleotide sequences and the predicted secondary structures of the resulting ribozymes are shown in Figure 1. Ribozymes directed against TNF α differ slightly from those used previously (10). For *in vitro* transcription, T7 RNA polymerase was incubated with PAGE-purified templates cleaved from plasmids as described by Promega. Transcription was primed with 7-methyl guanosine 5'triphospho 5'guanosine in all cases (21). Transcripts were treated with DNase (RNase-free, Boehringer), extracted with phenol, precipitated with ethanol, and then used for transfection.

Transfection of human cells with ribozymes

Human HL60 cells (ATCC CCL 240), growing at 37°C in RPMI medium 1640 supplemented with 20% (v/v) fetal calf serum, were transfected with liposomes containing ribozymes (lipofectin or DOTAP). At the end of the transfection period (20 h), cells were washed by centrifugation three times in Hank's buffered saline solution (Gibco), resuspended in RPMI supplemented with 20% fetal calf serum and incubated for various times at 37°C. In order to keep the cell number constant, the HL60 cells were plated at confluence.

Electrophoretic analysis of ribozyme stability

At various times after transfection, aliquots of cells were harvested by centrifugation, RNA was extracted according to reference (22), and the total RNA in the aliquot was subjected to electrophoresis in 15% polyacrylamide gels containing 7 M urea. To assure that RNA recovery was uniform, each aliquot was checked for nucleic acid content by measurement of absorbance at 260 nm. After electrophoresis, ribozyme RNA species were located in gels by autoradiography. Radioactive spots were excised, and radioactivity was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

In order to evaluate the effects of different 3'-terminal hairpins on ribozyme decay parameters, in this report we have performed decay kinetic analysis with mathematical treatment. We compared the intracellular stability of three hammerhead TNF α ribozymes that differ at their 3'-termini. One (RzA2) contained a bacteriophage T7 transcription terminator, one (RzC) contained the same terminator but with all AU and GU base pairs changed to GC base pairs, and one (RzB2) lacked a 3'-terminal hairpin. The nucleotide sequences and the predicted secondary structures of these ribozymes are shown in Figure 1. Human cell line HL60 was transfected with liposomes-treated ribozymes for 20 h. The cells at confluence were then incubated for various times, RNA was extracted, and RNA species were separated by gel electrophoresis. Figure 2 shows representative autoradiograms

Figure 1. Nucleotide sequences of ribozymes. Nucleotide sequences are shown for three hammerhead ribozymes directed against TNF- α mRNA. Ribozyme secondary structures shown were determined by the MFOLD program of Zuker (23).

following electrophoresis of RNA extracted from transfected cells. Ribozyme RNA was easily distinguished, and regions of gels containing ribozymes were excised for radioactivity determination. During the transfection period ribozyme recovery was constant. Thus the point at which exogenous ribozyme was removed was taken as zero time for kinetic analysis. As seen in Figure 2A, ribozymes were recovered from cells in decreasing amounts as incubation time increased, and as pointed out previously (10), those containing 3'-terminal hairpins were more stable than the ribozyme lacking a hairpin. Each of the TNF α ribozymes can be recovered in high yield even after many hours of incubation of HL60 at confluence. To establish that relative differences seen among ribozymes were not due to variation among batches of cells, we also transfected two ribozymes into the same cell culture simultaneously (Fig. 2B). Relative decay kinetics determined by single and double transfection were similar.

When radioactive ribozyme RNA was quantified and the results of several independent experiments combined, biphasic degradation was observed (Fig. 3). The data were well-fit by an expression that was the sum of two exponentials,

$$A(t) = C_F e^{-k_F t} + C_S e^{-k_S t} \quad (1)$$

in which A is the amount remaining at time t , k is the rate constant, and C is a constant. Subscripts denote the fast (F) and the slow (S) components of the degradation. The curves shown in Figure 3 were fit to the data by the method of exponential peeling (24) as follows. At long times ($t \geq 20$ h), when the fast

Figure 2. Intracellular stability of TNF- α ribozymes. (A) Recovery of ribozymes delivered individually to HL-60 cells. Cells were transfected with the indicated ribozyme complexed with lipofectin for 20 h, washed, and resuspended in growth medium for the indicated times. Aliquots of cells were harvested by centrifugation, RNA was extracted, RNA species were separated by gel electrophoresis, and radioactive RNA was detected by autoradiography as shown. Numbers above gel lanes indicate time (h) of incubation after transfection. Values of 100% represent about 1500 c.p.m. Symbols: open circles (RzC); closed circles (RzB2). (B) Recovery of ribozymes delivered as pairs to HL60 cells. Ribozymes and cells were treated as in panel (A) except that RzB2 and RzC were added simultaneously to cells. Numbers above gel lanes indicate time (h) of incubation after transfection. Arrows indicate position of ribozymes in gel. Symbols: open circles (RzC); closed circles (RzB2).

Table I. Kinetic parameters for ribozymes decay

Parameter	Ribozyme RzB2	RzA2	RzC	Rza*
C_F	33	18	29	29
K_F	0.39	0.17	0.24	0.36
r_F^2	0.997	0.954	0.980	0.979
C_S	65	80	80	67
K_S	0.0041	0.0040	0.0038	0.011
r_S^2	0.932	0.979	0.984	0.922

k_F and k_S are expressed in h^{-1} .

*integrase ribozyme lacking a terminal hairpin (8).

decay component had died away and only the slow decay remained, a curve was found that fit the data. This curve described the slow component of the biphasic degradation, and when this fitted curve was subtracted from the experimental data, the resulting values clustered around zero at long times. At short times ($0 \leq t \leq 20$ h), however, these residual differences deviated systematically from zero and described the fast decay component. An exponential curve was then fit to the fast decay data. The goodness of fit at each step of the peeling process was assessed by evaluating the resulting r^2 (25), a parameter that gives the fraction of observations that can be ascribed to the deterministic component of a process that fluctuates randomly about a causal relationship. Table 1 displays the values of the coefficients C_F , C_S , k_F and k_S found in each case, along with the goodness of fit parameter r^2 . In every case the coefficients C_F and C_S were positive and all fits were excellent.

The slow degradation phase has half-lives of 245 h (RzB2), 252 h (RzA2), and 267 h (RzC). These differences are not statistically significant. Thus the presence of a 3'-terminal hairpin has no significant effect on the time course of the slow degradation. However the rapid phase of degradation is slower for ribozymes containing 3'-terminal hairpins ($t_{1/2} = 3.0$ h (RzC) and 4.0 h (RzA2) than for the ribozyme (RzB2) lacking one ($t_{1/2} = 1.8$ h). The difference in degradation rate was significant at the 90% confidence level for comparisons of the presence and absence of 3'-terminal hairpins but not for comparison of the two ribozymes containing hairpins. Transfection experiments carried out at shorter time with RzA2 and RzC showed that RzA2 was slightly more stable than RzC with rapid phase half-lives of 3.3 and 2.0 h, respectively (data not shown); however, this difference was not statistically significant at the 90% confidence level. Thus we conclude that

Figure 3. Kinetics of hammerhead ribozyme degradation. Following transfection with radioactively-labeled ribozymes, HL60 cells at confluence were incubated for the indicated times, total RNA was extracted, and ribozymes were separated from other RNA by gel electrophoresis. The amount of ribozyme recovered was determined as shown in Figure 2. Symbols: RZA2 (dashed line, open circles); RZB-2 (solid line, triangles); RZC (solid line, closed circles). Error bars indicate the standard deviation of the mean in cases where it exceeded the size of the symbol; for RZA2 the error bars extend below the symbol and for RZC they extend above the symbol. Curves shown are best fits to the sum of two exponentials (see Text).

increasing the stability of the hairpin by replacing all AU and GU base pairs with GC base pairs does not increase intracellular ribozyme stability.

The binding of the endogenous protein(s) was found to be somewhat specific for TNF α ribozyme, since only a weak binding was observed with other ribozymes such as integrase ribozyme and interleukin-2 ribozyme. In all case a biphasic kinetic of degradation was observed (10,19). Calculation of the kinetic parameters (Table 1) showed that the slow phase of integrase ribozyme was three times more rapid than for the TNF α ribozymes. Thus confirming our previous data that endogenous proteins are providing protection for the TNF α ribozyme (19).

Stability of the ribozymes which we have examined so far is greater than, but in the same range as a RNA ribozyme examined by Taylor *et al.*, (20% of the ribozyme remained 48 h after transfection, ref. 26) and tRNA expressed from a gene injected into *Xenopus* oocytes (half-life of several days, ref. 27). In other examples, foreign RNA was considerably less stable. For example, antisense RNA in *Dictyostelium* decayed within minutes to hours (28,29), and a ribozyme embedded in a tRNA had a half life of less than a day (30).

In human cells, hammerhead ribozymes in general appear to be susceptible to at least two type of degradation as revealed by biphasic degradation decay. In this communication, we describe two possible explanations of this phenomenon. In one case the ribozyme enter cells in a form susceptible to degradation and are then converted to a protected form. Decay would be faster for the unprotected form than for the protected one ($k_u > k_p$) in this model, slow decay may arise from either partial protection or reversibility of the protection [binding of the protein to the TNF α ribozyme was found to be reversible (19)]. The overall

Figure 4. Schemes for biphasic ribozyme degradation. Two main general schemes can generate biphasic decay kinetics for ribozyme. In (A), the ribozyme enters the cells in an unprotected state characterized by a rapid rate of decay. Conversion to a protected state (ribozyme-protein complexes) slows the decay. Decay in the protected state may arise from protection being only partial or from it being reversible (19) to an unprotected state (free ribozyme). In (B) the ribozyme enters the cell in a protected state characterized by a slow decay rate. Conversion to a sensitive state speeds decay. Rate constants used in the text are indicated.

decay occurs as the sum of two exponential decay processes. For example suppose protection is irreversible ($k_- = 0$) and the protected form is more slowly degraded than the unprotected form ($k_u > k_p > 0$). If at time $t = 0$ all of the ribozyme molecules are unprotected ($u(0) = u_0$, $p(0) = 0$), then the total concentration of ribozyme, both protected and unprotected, at time t is

$$u(t) + p(t) = [u_0(k_u - k_p)/(k_u + k_+ - k_p)] e^{-(k_+ + k_u)t} + [u_0 k_+/(k_u + k_+ - k_p)] e^{-k_p t} \quad (2)$$

This is the sum of two exponentials, as all the coefficients are positive, and equation 2 has the same form as equation 1. (The general case ($k_- > 0$) is similar, although the exponents are more complex functions of the four rate constants (k_u , k_p , k_+ and k_-).

The ribozymes were delivered to the cells via liposomes, in the second case they could enter cells in a form protected from degradation and then be irreversibly converted to unprotected form ($p(0) = p_0$, $u(0) = 0$). Such a two-step degradative process would be expected if, for example, exonucleases were the major source of degradation and if the terminal hairpin provided protection from their action but was gradually removed. Such a model predicts that the total concentration of ribozyme at time t is expressed as the difference between two exponentials:

$$u(t) + p(t) = [k_d p_0/(k_d - k_u)] e^{-k_u t} - [k_u p_0/(k_d - k_u)] e^{-k_d t} \quad (3)$$

These two exponential terms always have opposite signs, because the concentration $u(t)$ of the unprotected form increases through deprotection and decreases through degradation. For the overall process, which coefficient is negative and which is positive depends on whether k_d or k_u is greater.

Since the data are well fit by the sum of two exponential in which C_F and C_S are both positive, they exclude two-step mechanisms (Fig. 4B) in which the major fraction of the incoming ribozyme is protected from degradation. Thus the terminal

hairpin, acting to block exonuclease activity, is unlikely to be a major factor in stability of TNF α ribozymes. This is consistent with the hairpin having relatively small effect (Fig. 3). Furthermore with *E. coli* cells we have found that an endonuclease is a major factor in ribozyme degradation (J.Y.Wang, unpublished observation). The binding of the protein to TNF α ribozyme was found to be reversible *in vitro* (19). Thus if the velocity of interconversion between free and protein bound-ribozyme is slower than that of degradation, the biphasic decay may also result from velocities of degradation and interconversion, respectively. A better understanding of the protective effects of hairpins and proteins may lead to a general way for improving intracellular ribozyme effectiveness.

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