The T Loop Structure Is Dispensable for Substrate Recognition by tRNase ZL

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The genome of almost every organism encodes the short form (tRNase ZS)1 and/or the long form (tRNase ZL) of tRNA 3′-processing endoribonucleases (tRNase Z, or 3′-tRNase; EC 3.1.26.11) are enzymes that remove 3′-trailers from pre-tRNAs. An about 12-base-pair stem, a T loop-like structure, and a 3′-trailer were considered to be the minimum requirements for recognition by the long form (tRNase ZL) of tRNase Z; tRNase ZL can recognize and cleave a micro-pre-tRNA or a hooker/target RNA complex that resembles a micro-pre-tRNA. We examined four hook RNAs containing systematically weakened T stems for directing target RNA cleavage by tRNase ZL. As expected, the cleavage efficiency decreased with the decrease in T stem stability, and to our surprise, even the hook RNA that forms no T stem-loop-directed slight cleavage of the target RNA, suggesting that the T stem-loop structure is important but dispensable for substrate recognition by tRNase ZL. To analyze the effect of the T loop on substrate recognition, we compared the cleavage reaction for a micro-pre-tRNA with that for a 12-base-pair double-stranded RNA, which is the same as the micro-pre-tRNA except for the lack of the T loop structure. The observed rate constant value for the double-stranded RNA was comparable with that for the micro-pre-tRNA, whereas the Kd value for the complex with the double-stranded RNA was much higher than that for the complex with the micro-pre-tRNA. These results suggest that the T loop structure is not indispensable for the recognition, although the interaction between the T loop and the enzyme exists. Cleavage assays for such double-stranded RNA substrates of various lengths suggested that tRNase ZL can recognize and cleave double-stranded RNA substrates that are longer than 5 base pairs and shorter than 20 base pairs. We also showed that double-stranded RNA is not a substrate for the short form of tRNase Z.

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1 The abbreviations used are: tRNase Z, tRNA 3′-processing endoribonuclease; tRNase ZS, a short form of tRNase Z; tRNase ZL, a long form of tRNase Z; pre-tRNA, precursor tRNA; nt, nucleotide(s); ATPγS, adenosine 5′-O-(3-thiotriphosphate).

† The on-line version of this article (available at http://www.jbc.org) contains three supplemental figures.

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A

B

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Fig. 1. Substrates of mammalian tRNase ZL contain the T loop structure. A, a secondary structure of human pre-tRNA<sup>5</sup>-Tr (red) with a target RNA. B, a secondary structure of the complex of a 3′-truncated human pre-tRNA<sup>5</sup>-Tr (red) with a target RNA. C, a secondary structure of the complex of a 5′-half tRNA<sup>5</sup>-Tr (red) with a target 3′-half tRNA. D, a secondary structure of human micro-pre-tRNA<sup>5</sup>-Tr, a secondary structure of the complex of an RNA heptamer (red) with a target RNA. E, a secondary structure of the complex of a hook RNA (red) with a target RNA. The T loop structures are shaded. Arrows denote the cleavage sites by tRNase ZL.

Experimental Procedures

Preparation of Various tRNase Z Proteins—Pig tRNase ZL was intensively fractionated from liver through several column chromatography procedures and further purified by glycerol gradient ultracentrifugation as described before (3, 24). The recombinant human tRNase ZL was produced from the pQE expression plasmids (Qiagen) in E. coli and purified with nickel-agarose as described (5).

RNA Synthesis—The micro-pre-tRNA MPTR<sub>1</sub>, six target RNAs ("T" series), and 28 sgRNAs were synthesized with T7 RNA polymerase (Takara Shuzo) from the corresponding synthetic DNA templates. The sequences of these RNAs are as follows: MPTR<sub>1</sub>, 5′-GGGCCAGACAGGUUUCGACUCCUGG-3′; T1H5, 5′-GGGCCAGCAGGUUUCGACUCCUGG-3′; T2L5, 5′-GGCAG-3′; T2L6, 5′-GGCAGC-3′; T2L7, 5′-GGCAGCA-3′; T2L8, 5′-GGCAGCAGU-3′; T2L9, 5′-GGCAGCAUUU-3′; T2L10, 5′-GGCAGCAUUU-3′; T2L11, 5′-GGCAGCAUUU-3′; T3L11, 5′-GGCAGCAUUU-3′; T3L12, 5′-GGCAGCAUUU-3′; T3L13, 5′-GGCAGCAUUU-3′; T3L14, 5′-GGCAGCAUUU-3′; T3L15, 5′-GGCAGCAUUU-3′; T3L16, 5′-GGCAGCAUUU-3′; T3L17, 5′-GGCAGCAUUU-3′; T3L18, 5′-GGCAGCAUUU-3′; T3L19, 5′-GGCAGCAUUU-3′; T3L20, 5′-GGCAGCAUUU-3′; T4L16, 5′-GGCAGCAUUU-3′; T4L17, 5′-GGCAGCAUUU-3′; T4L18, 5′-GGCAGCAUUU-3′; T4L19, 5′-GGCAGCAUUU-3′; T4L20, 5′-GGCAGCAUUU-3′; T4L21, 5′-GGCAGCAUUU-3′; T4L22, 5′-GGCAGCAUUU-3′; T4L23, 5′-GGCAGCAUUU-3′; T4L24, 5′-GGCAGCAUUU-3′; T4L25, 5′-GGCAGCAUUU-3′; T4L26, 5′-GGCAGCAUUU-3′; T4L27, 5′-GGCAGCAUUU-3′; T4L28, 5′-GGCAGCAUUU-3′; T4L29, 5′-GGCAGCAUUU-3′; T4L30, 5′-GGCAGCAUUU-3′. The transcription reactions were carried out under the conditions recommended by the manufacturer (Takara Shuzo), and the transcribed RNAs were purified by denaturing gel electrophoresis.

The RNA transcripts for MPTR<sub>1</sub> and the six target RNAs were subsequently labeled with fluorescein according to the manufacturer's protocol (Amersham Biosciences) (5). Briefly, after the removal of the 5'-phosphates of the transcribed RNAs with bacterial alkaline phosphatase (Takara Shuzo), the RNAs were phosphorylated with T4 polynucleotide kinase (Takara Shuzo) and ATPγS. Then a single fluorescein moiety was appended onto the 5'-phosphoribonucleotide site. The resulting fluorescein-labeled RNAs were gel-purified before assays.

In Vitro RNA Cleavage Assays—The in vitro RNA cleavage assays for the fluorescein-labeled MPTR<sub>1</sub> were performed with tRNase ZL in a mixture (6 μl) containing 10 mM Tris-HCl (pH 7.5), 1.5 mM dithiothreitol, and 3.3 mM MgCl₂ (15). The in vitro cleavage assays for the fluorescein-labeled target RNAs were carried out in the presence of the unlabeled sgRNAs using various tRNase Zs under the same conditions as above (15). After resolution of the reaction products on a 10 or 20%
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The T Stem/Loop Structure Is Important but Dispensable for Substrate Recognition by tRNase ZL—We have shown how important the T stem-loop structure is for substrate recognition by tRNase ZL by analyzing heptamer-directed cleavages of the 3'-half-tRNA\(^{AVE}\) containing systematically weakened T stems (19). In this analysis, we have observed that the cleavage efficiencies decrease with the decrease in the T stem stability, and we have not been able to detect any cleavage of the 3'-half-tRNA\(^{AVE}\) with no T stem. To corroborate these observations, we examined four hook RNAs, T1H5, T1H4, T1H3, T1H0, containing systematically weakened T stems for directing target RNA cleavage by tRNase ZL (Fig. 2A). This series of the T stems was the same as that used in the heptamer-directed RNA cleavage assays.

As expected, the target RNA T-1 was most efficiently cleaved by tRNase ZL in the presence of T1H5, and the cleavage efficiency decreased with the decrease in the T stem stability (Fig. 2B). The cleavage took place after a nucleotide corresponding to the discriminator. To our surprise, even T1H0, which had no T stem, directed slight cleavage of the target RNA. Furthermore, tRNase ZL cut the target slightly in the presence of the RNA heptamer T1Hep corresponding to the 5'-portion of the acceptor stem. These results suggest that the T stem-loop structure is important but dispensable for substrate recognition by tRNase ZL. The discrepancy between this and previous results may be because of the difference in the assay conditions; this analysis was performed in the presence of 3.3 mM MgCl\(_2\) instead of 3.3 mM spermidine. We found that generally, spermidine is much less effective in supporting tRNase ZL cleavage reactions than Mg\(^{2+}\) (Supplemental Fig. 1).

The T Loop Is Dispensable—We were curious about which portion of the T stem/loop is important for the tRNase ZL activity. A previous study has shown that several double-stranded RNA substrates are recognizable by tRNase ZL (20). In this case, however, formation of alternative conformers that look like pre-tRNAs retaining the T loop-like structures appears to be required. On the other hand, it has been shown that pre-tRNAs lacking distal base pairings of the T stem are not substrates (23). These observations suggest that the T stem may be more important for substrate recognition by tRNase ZL than the T loop.

To address this issue more precisely, we compared the cleavage reaction for a micro-pre-tRNA with that for a 12-base-pair double-stranded RNA, which is the same as the micro-pre-tRNA except for the lack of the T loop structure (Fig. 3A). The micro-pre-tRNA MPTR1 was synthesized with T7 RNA polymerase and 5'-end-labeled with fluorescein. Both strands of the double-stranded RNA were separately transcribed by T7 RNA polymerase, and only the target RNA strand T-6 was 5'-end-labeled with fluorescein and annealed with the unlabeled sgRNA strand T6L1. Time course assays showed that there is no significant difference in cleavage efficiency between both substrates (Fig. 3B). Although pig tRNase ZL was used to
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compare these data with previous data, which have shown that the cleavage efficiency of the micro-pre-tRNA is comparable with that of a natural full-length pre-tRNA (23), similar results were also obtained using recombinant human tRNase ZL (data not shown). As expected, each cleavage occurred primarily after a nucleotide corresponding to the discriminator (Fig. 3B). The reason why a simple double-stranded RNA was not cleaved by tRNase ZL in the previous study (20) would be because spermidine was used instead of Mg\(^{2+}\) as mentioned above.

We tried to determine the kinetic parameters \(K_m\) and \(k_{cat}\) for the reaction of the double-stranded RNA, but reliable values were not obtained due to large standard deviations. Thus, we measured the observed rate constant \(k_{obs}\) for each reaction under single turnover conditions instead of \(K_m\) and \(k_{cat}\). First, we determined the dissociation constant of the double-stranded RNA by gel-shift analysis. Using this estimated \(K_d\) value (50 nM), we calculated an effective concentration of the double-stranded RNA substrate. The \(k_{obs}\) value (0.013 min\(^{-1}\)) for the double-stranded RNA T6L1/T-6 was comparable with the value (0.023 min\(^{-1}\)) for the micro-pre-tRNA MPTR1 (Table I). Taken together, present and previous results suggest that the T loop structure is not indispensable for substrate recognition by tRNase ZL.

In addition, we measured dissociation constants of the tRNase ZL substrate complexes by gel-shift assays. The \(K_d\) value (420 nM) for the complex with the double-stranded RNA was 130-fold higher than that (3.2 nM) for the complex with the micro-pre-tRNA (Table I), indicating that the interaction between the T loop and the enzyme exists and helps make the enzyme/substrate complex.

tRNase ZL Can Recognize and Cleave Double-stranded RNA Substrates That Are Longer than 5 Base Pairs and Shorter than 20 Base Pairs—To determine the minimum and maximum lengths of double-stranded RNA substrates that are recognizable and cleavable by tRNase ZL, we examined double-stranded RNA substrates of various lengths for tRNase ZL cleavage. The first series of the substrates was composed of the target T-2 and one of the eight sgRNAs T2L5-T2L12, which are complementary to T-2 (Fig. 4A). These substrates were assayed, and cleavage efficiencies were quantitated and plotted against the length of double-stranded RNA (Fig. 4, B and C). tRNase ZL cleaved the target T-2 with the aid of each sgRNA with the exception of T2L5, and the double-stranded RNA complexes with the longer sgRNAs were better substrates. In each case, the primary cleavage site was located after a nucleotide corresponding to the discriminator (Fig. 4B).

In the second series, the target RNA T-3 was tested for tRNase ZL cleavage in the presence of the five sgRNAs T3L11, T3L12, T3L13, T3L14, and T3L16 (Fig. 5A). The cleavage efficiency increased with the increase in sgRNA length (Fig. 5, B and C). In the third set of substrates, 12-, 16-, 18-, 20-, 25-, and 30-base-pair RNAs were assayed using the target T-4 and the five sgRNAs, T4L12, T4L16, T4L18, T4L20, T4L25, and T4L30 (Fig. 6A). The 12-base-pair RNA substrate was cleaved relatively well, and the 16- and 18-base-pair substrates were barely cleaved (Fig. 6, B and C). The substrates longer than 18 base pairs were not substrates for tRNase ZL in these series, cleavages occurred primarily after a nucleotide corresponding to the discriminator (Figs. 5B and 6B).

![Fig. 3. tRNase ZL cleavage reactions for substrates with and without the T loop.](http://www.jbc.org/)

**Table I.** Observed rate constants and dissociation constants for tRNase ZL

| Substrate | \(k_{obs}\) | \(K_d\) |
|-----------|-----------|-----|
| Micro-pre-tRNA | 0.023 ± 0.0011 | 3.2 ± 0.85 |
| Double-stranded RNA | 0.013 ± 0.0009 | 420 ± 32 |

a Each fluorescein-labeled substrate (1.6 pmol) was incubated with pig tRNase ZL (4.2 pmol) at 37 °C for 4 min. The values are chemical cleavage reaction rates per a substrate molecule. Data are the means ± S.D. of three independent experiments.

b Data are the means ± S.D. of three independent experiments.
Simple Complementary RNA Molecules Can Be Used for Targeted RNA Cleavage by tRNase ZL—Because tRNase ZL can recognize and cleave double-stranded RNA substrates that are longer than 5 base pairs and shorter than 20 base pairs and that the enzyme is the most active for 12–16-base-pair RNAs.

**DISCUSSION**

Interaction of tRNase ZL with a Double-stranded RNA Substrate—Previously, we have shown that micro-pre-tRNAs or micro-pre-tRNA-like complexes, which consist of the T stem-loop and the acceptor stem plus a 3′-trailer, are the smallest substrates for tRNase ZL (19–23). In this report, however, we found that the T loop structure is important but not indispensable. Therefore, the genuine minimum substrate of tRNase ZL is considered to be composed of the T stem and the acceptor stem plus a 3′-trailer.

The importance of the T loop structure for recognition is indicated by the much lower $K_d$ value for the substrate with the T loop than for the substrate without the T loop. From the difference between these $K_d$ values (Table I), we estimated the free energy $\Delta G$ for the enzyme/T loop interaction to be $-2.7$ kcal/mol. We guess that a couple of weak chemical bonds would be involved in this interaction.

The double-stranded RNA substrates cleavable by tRNase ZL ranged from 6 to 18 base pairs, and the enzyme was the most active for 12–16-base-pair RNAs. The double-stranded RNA substrates less than 6 base pairs would be too small to stably bind to the enzyme, and those longer than 18 base pairs would be too long to fit the T stem end into the enzyme pocket. The double-stranded RNA substrates of 12–16 base pairs would...
be able to mimic micro-pre-tRNA most well especially at the end of the T stem. The reason why the 11-base-pair RNA substrate containing the 100-nt target strand was cleaved by tRNase ZL may be because the single strand RNA portion is floppy enough to avoid steric hindrance in the enzyme pocket, whereas the double-stranded RNA portion is too rigid to dodge the hindrance.

Cleavage Site Selection in Double-stranded RNA Substrates—There exists a general rule for cleavage site selection by tRNase ZL with respect to full-length pre-tRNAs (11). Cleavage of pre-tRNAs containing a total of N base pairs (N < 12) in the acceptor stem and the T stem occurs after 12 – N and 13 – N nucleotides 3’ to the discriminator nucleotide, whereas cleavage of pre-tRNAs with a total of N base pairs (N ≥ 12) occurs after the discriminator. For example, the pre-tRNA R-ASD2 containing a total of 10 base pairs was cleaved after 2 and 3 nucleotides 3’ to the discriminator nucleotide, and cleavage of the pre-tRNA R-ASA2 containing a total of 14 base pairs occurred after the discriminator (11). This rule needs to be modified with respect to micro-pre-tRNAs containing a total of N base pairs (N < 12) (23). Cleavage occurs at multiple sites fluctuating between the discriminator nucleotide and 12 – N or 13 – N nucleotides 3’ to the discriminator.

We have proposed a model to explain this difference, taking into account the presence of “bottom-half” tRNA (23). Pre-tRNAs would be prevented from moving along the “top-half” tRNA binding domain on tRNase ZL due to the bottom-half tRNA constraints, whereas micro-pre-tRNAs would be able to slide along this domain. The cleavage site would change depending on the fashion of enzyme/substrate interaction. When the distal acceptor stem is located in the vicinity of the catalytic domain, the cleavage would occur after the discriminator nucleotide. On the other hand, when the T loop is arranged at its original binding domain, the cleavage would occur after 12 – N and 13 – N nucleotides 3’ to the discriminator.

The present study showed that cleavages of the double-stranded RNA substrates occur primarily after the discriminator. This observation can be interpreted in the context of the above model; that is, the distal acceptor stem of double-stranded RNA would be preferentially arranged near the catalytic domain due to the lack of the T loop regardless of the N number.

Cleavages of the RNA complexes T1H4/T-1 and T1H3/T-1, which contain the T loop but lack one or two proximal T stem base pairings, occur after the discriminator (Fig. 2). This is consistent with the previous observation that micro-pre-tRNAs containing proximal base pair disruptions behave like a substrate with 12 intact base pairs and that tRNase ZL can select the cleavage site in a fashion similar to that of their original micro-pre-tRNA (23).

An Extra Physiological Function of tRNase ZL?—The human genome contains both tRNase ZS and tRNase ZL genes, raising an interesting question of whether the short and long forms play different roles in the cells. One may be for nuclear tRNA processing, and the other may be for mitochondrial processing. Alternatively, tRNase ZL, which has the extra N-terminal region that is not essential for the RNA cleaving catalytic reaction, might have an additional role for some RNA metabolism.

To elucidate their possible differential roles, we have recently investigated whether the long and short forms have...
FIG. 6. The tRNase ZL reactions for T-4-containing double-stranded RNA substrates. A, secondary structures of the sgRNA/target RNA complexes T4L12–30/T-4. The last number of each sgRNA T4L12–30 denotes its nucleotide length, and its common and extended sequences are shown in capital and small red letters, respectively. An arrow indicates a cleavage site by tRNase ZL. B, pig tRNase ZL (2 pmol) was incubated with the fluorescein-labeled T-4 (2 pmol) in the absence or presence of 29 pmol of T4L12, T4L16, T4L18, T4L20, T4L25, or T4L30 at 52 °C for 15 min. The cleavage products were analyzed on a 20% polyacrylamide-8 M urea gel. The target and the primary 5’-cleavage product (5’P) are indicated by a bar and an arrow, respectively. L denotes an alkaline ladder of the fluorescein-labeled T-4. Although a part of the ladder may not be viewed clearly, each band was visible from the original picture. The substrate band of the precise length among the 3’-heterogeneous transcript bands from the intrinsic property of T7 RNA polymerase was identified by comparison with a size standard. C, percentages of T-4 cleavages in the presence of the sgRNAs of various lengths are shown. Data are the means ± S.D. of three independent experiments.

FIG. 7. The in vitro cleavage assays for a long double-stranded RNA substrate. A, secondary structures of the RNA complexes between the target T-5 and the sgRNAs T5L1–3. Another complex of T5L2 with the target T-5, T5L2/T-5*, is also shown, which can explain an unexpected T-5 cleavage. The sgRNAs are shown in red. An arrow indicates a cleavage site by tRNase ZL. B, the fluorescein-labeled T-5 (2 pmol) was incubated with pig tRNase ZL (2 pmol) in the absence or presence of 29 pmol of T5L1–3 at 52 °C for the indicated time period. The cleavage products were analyzed on a 10% polyacrylamide-8 M urea sequencing gel. A bar and arrows with parenthesized numbers indicate the target and the primary 5’-cleavage products (5’P) directed by the sgRNAs with the same numbers, respectively. An asterisk denotes the unexpected cleavage product that appears only in the presence of T5L2. L, an alkaline ladder of the fluorescein-labeled T-5; M, 93- and 77-nt size markers.
different preferences for RNA substrates and have found that indeed tRNase ZL recognizes and cleaves a wider range of substrates than tRNase ZS and that this versatility is attributed to the N-terminal half domain of tRNase ZL (15). It has been shown that in HeLa cells, tRNase ZL physically interacts with the γ-tubulin complex (25), suggesting that tRNase ZL exists in cytoplasm as well as nuclei where the tRNA 3′-processing occurs. This ubiquity of tRNase ZL would extend its niches in cellular RNA metabolism. In this report, we demonstrated that tRNase ZL shows further versatility in substrate recognition; the enzyme can recognize and cleave double-stranded RNA substrates. Taken together, these observations support the notion that tRNase ZL may play extra roles in other cellular RNA metabolism.

Although at present we do not have enough information to specify which other RNA metabolism tRNase ZL is involved in, tRNase ZL may work in the four different manners as follows: (i) RNase 65 with the aid of 3′-truncated tRNAs (16, 17), (ii) RNA cutters that recognize pre-tRNA-like complexes of targets with microRNAs (20), (iii) 4-nt RNA cutters in the presence of hook RNAs (21), and (iv) double-stranded RNA cutters with complementary sgRNAs. A huge number of microRNAs, the length of which is ~21–23 nt, are found in mammalian cells (26), and other small non-coding RNAs might also exist. These small RNA molecules could work as hook RNAs or complementary sgRNAs.

Although human tRNase ZL is the product of the candidate prostate cancer gene ELAC2 (1, 3), we have no information on how the mutations in the tRNase ZL gene can cause human prostate cancer. The tRNase ZL variants containing amino acid substitutions from such mutations show only trivial differences in both RNA 3′-processing activity and RNase 65 activity (27). These trivial changes might be sufficient to alter the whole gene expression pattern in prostate cells to the cancerous state. Alternatively, activities of the other RNA cutters from tRNase ZL might be affected significantly by these mutations.

**Targeted Cleavage of Undesirable RNA by tRNase ZL with the Aid of 11-nt Complementary sgRNA—Mammalian tRNase ZL can cleave any RNA at any site under the direction of sgRNA in vitro (18–21). sgRNAs can be as short as heptamers, which are much smaller than small interfering RNAs of ~21 nt (19). Together with such flexibility in substrate recognition, the ubiquity and the constitutive expression of tRNase ZL have suggested that this enzyme can be utilized for specific cleavage of cellular RNAs by introducing appropriate sgRNAs into living cells. Indeed, the expression of exogenous and endogenous genes has been down-regulated by appropriate sgRNAs that were introduced into mammalian culture cells as expression plasmids or synthetic 2′-O-methyl RNAs (22). The discovery that the 11-nt complementary sgRNA can direct tRNase ZL cleavage of the long RNA target may lead us to examine the efficacy of simple complementary sgRNA on down-regulation of cellular gene expressions. Although this type of sgRNA would also work in the cells, the present data suggest that its guiding efficiency and substrate specificity would be lower than those of sgRNA that can form complexes containing a T loop-like structure with target RNAs. Thus, we would prefer the latter type of sgRNA for destroying undesirable cellular RNAs.

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