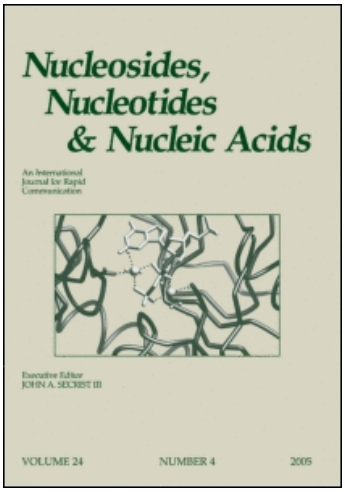

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Ribonuclease Activity of the Peptides with Alternating Arginine and Leucine Residues Conjugated to Tetrathymidilate

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ABSTRACT

RNA cleaving conjugates have been prepared by attachment of oligodeoxyribonucleotide TTTT to peptides containing arginine, leucine, proline and serine residues. The highest activity was displayed by the conjugates containing peptides with alternating arginine and leucine residues $(LR)_4$G-amide. Ribonuclease activity of the conjugates pep-T_4 decreases in the order T_4-$(LR)_4G > T_4$-$(LR)_2G > T_4$-(LLRR)2G > T_4-$(LR)_2$PRLRG > S_2R_3-Hmda-$T_4 \geq R_5 \neq (LR)_3$. According to CD spectra, the free peptide $(LR)_4$G-amide in water solution at neutral pH and physiological ionic strength has no pronounced secondary structure whereas conjugated to oligonucleotide it acquires a folding similar to α-helix.

Key Words: Artificial ribonucleases; Oligonucleotide-peptide conjugates.

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INTRODUCTION

In attempts to develop small artificial ribonucleases a number of peptides was synthesized and some of them were shown to display ribonuclease activity (for reviews see Refs. [1,2]). One of the identified RNA-cleaving peptide structures is the polypeptide built of alternating basic and hydrophobic amino acids.[3–5] Conjugation of short peptides with repeating arginine-leucine units to oligodeoxyribonucleotides resulted in considerable enhancement of the ribonuclease activity.[6–8] In the present study we investigated ribonuclease activity of different peptides conjugated to tetrathymidilate.

EXPERIMENTAL

Materials

T4 polynucleotide kinase was purchased from Fermentas (Lithuania), [γ-^{32}P]ATP (3000 Ci/mmol) was from Biosan Co. (Russia), RNase T1 was from Boehringer Mannheim (Germany). RNA HIV-1 (123–218 nt) was prepared by in vitro transcription with T7 RNA polymerase using *Fok I*-linearized plasmid pHIV-1.[9] HIV-1 RNA was labeled at the 5′-terminus with [γ-^{32}P]ATP and T4 polynucleotide kinase as described in Ref. [10]. Oligopeptides $(LR)_nG$ (n = 4, 3, 2), R_5, $(RRLL)_2G$, $(LR)_2PRLRG$, S_2R_3 were synthesized by Dr. A. Kolobov (State Research Institute of Highly Pure Biopreparations, St.-Petersburg, Russia).

Oligodeoxyribonucleotide pTTTT was synthesized by standard solid-phase phosphoramidite procedure on automatic synthesizer ASM-700 (Biosset, Russia). Oligonucleotide-peptide conjugates were synthesized according to described protocols[11] and purified by reverse phase HPLC on LiChrosorb RP-18 columns. The homogeneity of the conjugates was determined according to analysis by electrophoresis in 15% PAGE/8 M urea gel followed by ''Stains-all'' staining was 95–98%.

RNA Cleavage by Oligonucleotide-Peptide Conjugates

Reaction mixtures (10 μl) containing 50000 cpm [^{32}P]-labeled 96 nt long fragment of HIV-1 RNA (10^{-7} M), one of the conjugates at concentration 50 μM, 50 mM Tris-HCl, pH 7.0, 0.2 M KCl, 1 mM EDTA, and 100 μg/ml RNA carrier (total tRNA *Escherichia coli*) were incubated at 37°C for 9 h. The reaction was quenched by precipitation of RNA and RNA fragments with 2% lithium perchlorate solution in acetone (150 μl). RNA was collected by centrifugation and dissolved in gel-loading buffer (6 M urea, 0.025% bromophenol blue, 0.025% xylene cyanol). RNA cleavage products were resolved in 12% polyacrylamide/8 M urea gel using TBE × 1 (100 mM Trisborate, pH 8.3, 2 mM EDTA,) as a running buffer. To identify cleavage sites, imidazole ladder[12] and G-ladder[13] produced by partial RNA cleavage with 2 M imidazole, pH 7.0, and RNase T1 were run in parallel. Substrate and cleavage products were quantitated using Bio-Rad Molecular Imager FX. The total extent of RNA cleavage as well as the extents of cleavage at any given site were determined as a ratio of radioactivity measured in the RNA fragment(s) to the total radioactivity applied on the lane.

RESULTS AND DISCUSSION

The recent study of conjugates containing peptide $(LR)_4G$ and different oligonucleotides revealed that conjugates displayed ribonuclease activity and cleaved RNA substrate at Pyr-A and G-X motifs.[7,8,14] The tetrathymidylate conjugate was one of the most active among the compounds. In the present work we varied the sequence and length of the peptide part of the tetrathymidylate based conjugates to identify the optimal peptide structure.

Conjugates of tetrathymidylate and peptides $(LR)_nG$ (n = 4, 3, 2), R_5, $(RRLL)_2G$, $(LR)_2PRLRG$ (Fig. 1) were synthesized according to published protocol[11] by formation of the phosphamide bond between the 5′-terminal phosphate of the oligonucleotide and α-aminogroup of N-terminal amino acid of the peptide. In the case of peptide S_2R_3, the oligonucleotide was attached to its C-terminal arginine amide residue using hexamethylenediamine linker.

Ribonuclease activity of the conjugates was assayed in experiments with 5′-[^{32}P]-labeled fragment of HIV-1 RNA under physiological conditions as described in experimental part (Fig. 2). Control experiments showed that neither oligonucleotide, nor the peptides or their equimolar mixture display any detectable ribonuclease activity

a)

Oligonucleotide (5' - 3')	*Peptide (N - C)*
pTTTT	$(LR)_4G$-amide
	RRRRR-amide
	$(RRLL)_2$-G-amide
	$(LR)_2PRLR$-G-amide
	Ac-SSRRR-NH-Hmda(*)

b)

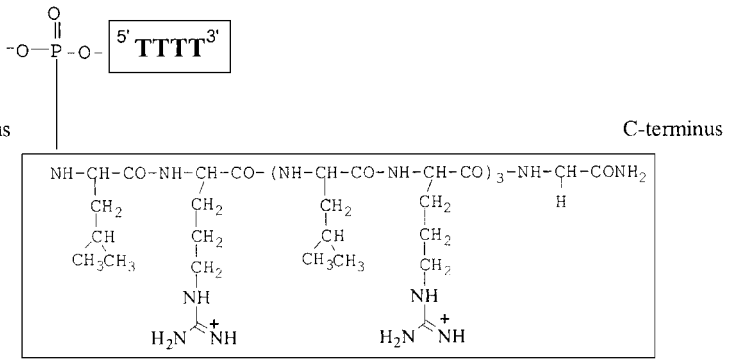

Figure 1. The structure of oligonucleotide-peptide conjugates. (a) Sequences of the oligonucleotide and peptides used in the study. (b) As an example the structure of oligonucleotide-peptide conjugate T_4-$(LR)_4G$-amide is shown. All peptides except for R_5 and S_2R_3 contain glycine-amide at the C-terminus. (*) Peptide S_2R_3 contains hexamethylenediamine group at the C-terminus.

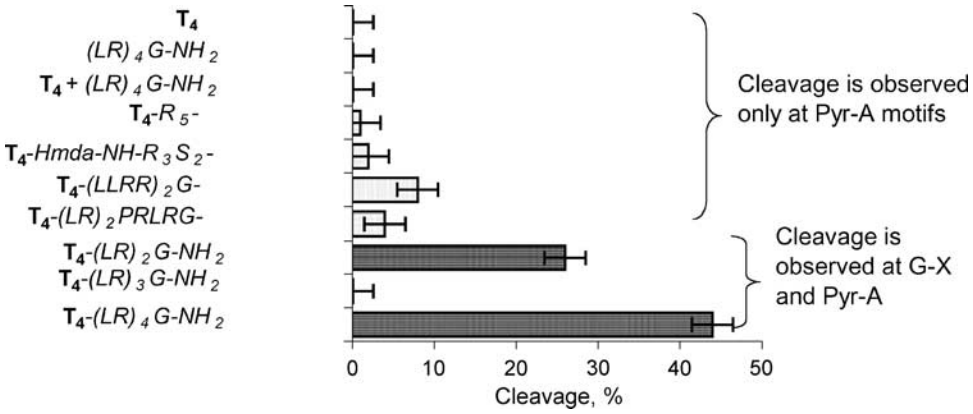

Figure 2. The influence of peptide length and sequence on efficacy of RNA cleavage. Assay conditions: 5′-[^{32}P]-HIV-1 RNA (96 nt long) was incubated in 50 mM Tris-HCl, pH 7.0, containing 200 mM KCl, 1 mM EDTA, 0.1 mg/ml RNA carrier, in the presence of conjugates (50 μM each) at 37°C for 9 h. Cleavage extent, %- total RNA depolymerization.

under the assay conditions. Conjugate **T$_4$**-*R*$_5$ containing only arginine residues displays no ribonuclease activity. Conjugate **T$_4$**-*R*$_3$*S*$_2$ with arginine and serine residues displayed only trace activity (cleavage extent 1–2%). The most effective conjugate contains *nona*peptide consisting of alternating arginine and leucine residues $(LR)_4G$ and cleaves 55% RNA under the assay conditions. Substitution of leucine residue in the third position from N-terminus of the peptide $(LR)_4G$ by proline strongly inhibited ribonuclease activity: 55% and 3% RNA cleavage for conjugates **T$_4$**-$(LR)_4G$ and **T$_4$**-$(LR)_2PRLRG$, respectively. Thus, the ribonuclease activity of the compounds is provided essentially by the peptide component.

All the tested conjugates except for **T$_4$**-$(LR)_nG$ cleave RNA exceptionally at Pyr-A motifs. Conjugates **T$_4$**-$(LR)_nG$ (n = 4, 2) display complex cleavage specificity: these conjugates cleave RNA at G-X sequences, and less efficiently at Pyr-A motifs. Previously we observed G-X specificity of cleavage only in the case of the conjugate of oligonucleotide pCCAAACA with the peptide $(LR)_4G$.[8] The G-X specificity can be explained by specific interaction between the oligonucleotide part of the conjugates (TTTT or CCAAACA) with peptide $(LR)_4G$. This interaction may in some way promote the guanidinium groups of arginine residues to adapt a conformation optimal for binding with G nucleobase thus providing tight contact and efficient cleavage at G-X phosphodiester bonds.

The influence of the peptide length on the ribonuclease activity was studied in experiments with the conjugates T_4-$(LR)_nG$ (n was 4, 3 and 2). Decrease of the peptide length from 4 to 3 units (LR) entirely abolished the cleavage activity whereas conjugate containing two (LR) units displayed detectable but low activity as compared to conjugate **T$_4$**-$(LR)_4G$. Probably in the case of conjugates containing peptides built of 2 or 4 leucine-arginine units an optimal intramolecular conformation of the amino acids side chains is achieved which is required the ribonuclease activity.

CD spectra of the peptide $(LR)_4G$ and its conjugate with tetrathymidylate show that free peptide $(LR)_4G$-amide in water solution at neutral pH and physiological ionic

strength is unfolded (a single band with minimum ellipticity at 195 nm) and its conformation is affected by the environment. The CD spectrum of the peptide conjugated to oligonucleotide is represented by the curve with minimum ellipticity at 200 nm with shoulder at ~220 nm indicating that the peptide adopt some regular secondary structure upon the conjugation. Apparently the ribonuclease activity of peptidyloligonucleotides is provided by the peculiar structure of the conjugate and the conjugated oligonucleotide affects the peptide folding.

Thus, for the first time an efficient artificial ribonuclease consisting of short oligonucleotide (T_4) and peptide capable of cleaving RNA at G-X sequences was developed. Results of the experiments indicate that the ribonuclease activity of the conjugate is provided by the regular structure of the peptide part of the conjugate and stabilized by the conjugated oligonucleotide.

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