HIV-1 TAR RNA Recognition by an Unnatural Biopolymer

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Small peptides with unnatural backbones that can bind with high affinity to any predetermined RNA structure would provide useful tools in molecular biology and medicine. Recently, minor-groove-binding polyamide ligands have been designed for sequence-specific recognition of DNA.[1−3] RNA molecules can fold into extensive structures containing regions of double-stranded duplex, hairpins, internal loops, bulged bases, and pseudo-knotted structures.[4] The complexity of RNA structure makes it difficult to design ligands for sequence-specific RNA recognition. RNA−protein interactions are important in many cellular functions, including transcription, RNA splicing, and translation. One example of such interactions is the mechanism of *trans*-activation of human immunodeficiency virus type 1 (HIV-1) gene expression that requires the interaction of Tat protein with the *trans*-activation responsive region (TAR) RNA, a 59-base stem−loop structure located at the 5′-end of all nascent HIV-1 transcripts.[5] An RNA-binding unnatural biopolymer consisting of chiral aminocarbonate monomers linked via a carbamate backbone was synthesized by solid phase methods[6] and tested for TAR RNA binding. This tat-derived unnatural biopolymer can specifically bind TAR RNA with high affinities. Site-specific photo-cross-linking experiments using a photoactive analog (4-thiouracil) containing TAR RNA revealed that the unnatural biopolymer interacts with RNA in the major groove. The oligocarbamate−RNA complexes were stable to proteolytic digestion. These results identify a new class of unnatural peptides for structure-specific recognition of RNA.

The promoter of HIV-1, located in the U3 region of the viral long terminal repeat (LTR), is an inducible promoter which can be stimulated by the *trans*-activator protein, Tat.[5] As in other lentiviruses, Tat protein is essential for *trans*-activation of viral gene expression.[5,7−9] A number of studies showed that Tat-derived peptides which contain the basic arginine-rich region of Tat are able to form *in vitro* complexes with TAR RNA.[10−12] We synthesized a tat-derived oligocarbamate (Figure 1) containing the basic-arginine-rich region of full length Tat protein by solid phase peptide synthesis methods. The oligocarbamate backbone consists of a chiral ethylene backbone linked through relatively rigid carbamate groups.[6] After HPLC purification and characterization by mass spectrometry, the oligocarbamate was tested for TAR RNA binding (Figure 2). The tat-derived oligocarbamate was able to bind TAR RNA and failed to bind a mutant TAR RNA without the bulge residues.

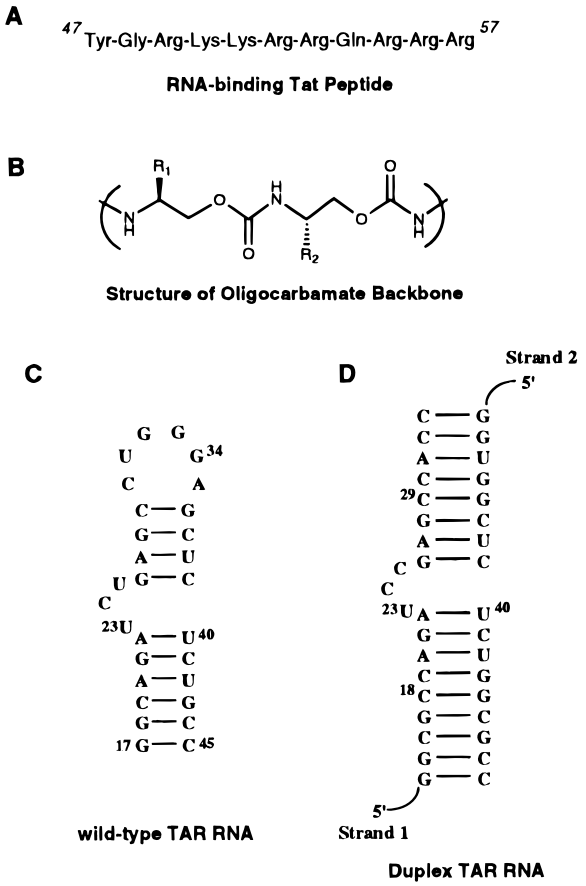

Figure 1. (A) The Tat-derived peptide, amino acids 47−57, contains the RNA-binding domain of Tat protein. (B) Structure of the oligocarbamate backbone. Sequence of the oligocarbamate corresponds to the Tat peptide shown in A. Tat-derived oligocarbamate was synthesized on an ABI 431 peptide synthesizer by using *N*-α-Fmoc-protected *p*-nitrophenyl carbonate monomers.[6] After cleavage from the resin, the oligocarbamate was purified by HPLC on a Zorbax 300 SB-C_8 column.[15] The mass of fully deprotected, and purified oligocarbamate was confirmed by FAB mass spectrometry: 1831.3 (M + H). (C) Secondary structure of wild-type TAR RNA used in this study. Wild-type TAR RNA spans the minimal sequences that are required for Tat responsiveness *in vivo*[18] and for *in vitro* binding of Tat-derived peptides.[10] Wild-type TAR contains two non-wild-type base pairs to increase transcription by T7 RNA polymerase. (D) The secondary structure of the designed duplex TAR RNA used in photo-cross-linking experiments.[15] Duplex RNA contains the nucleotides C18−C29 and G36−G44 from the wild-type TAR RNA sequence and extra flanking base pairs to enhance hybridization of two RNA strands. Numbering of nucleotides in the duplex TAR RNA corresponds to their positions in wild-type TAR RNA. All RNAs were prepared by *in vitro* transcription.[15,19] For transcription reactions (20 μL) containing 8.0 pmol of template DNA, 40−60 units of T7 polymerase (Promega) was used. For the synthesis of 4-thioU labeled RNA, UTP was replaced with 4-thioU-TP (4 mM, final concentration) in the transcription buffer. 4-ThioUTP was synthesized according to the method of Stade *et al.*[20] RNA purification and labeling were carried out as described earlier.[15]

Equilibrium dissociation constants of the oligocarbamate−TAR RNA complexes were measured using direct and competition electrophoretic mobility assays.[13] Dissociation constants were calculated from multiple sets of experiments which showed

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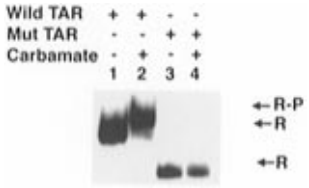

Figure 2. Electrophoretic mobility shift analysis for the Tat-derived oligocarbamate binding to wild-type (wild) and trinucleotide bulge mutant (mut) TAR RNA. RNA and RNA−oligocarbamate complexes are indicated as R and R−P, respectively. ^{32}P-5′-end labeled TAR RNAs were heated to 85 °C for 3 min and then cooled to room temperature in TK buffer (50 mM Tris-HCI (pH 7.4), 20 mM KCI, 0.1% Triton X-100). The oligocarbamate was added alone or along with the competitor RNAs (wild-type TAR or mutant TAR) preheated in TK buffer at 85 °C for 3 min and cooled to room temperature. The oligocarbamate−RNA binding reactions were carried out at room temperature for 1 h and stopped by adding 30% glycerol. The oligocarbamate−RNA complexes were resolved on a non-denaturing 12% acrylamide gel and visualized by autoradiography or Phosphorimaging.

that the oligocarbamate binds TAR RNA with a K_d of 1.13 μM. To compare the RNA-binding affinities of the oligocarbamate to natural peptide, we synthesized a tat-derived peptide (Tyr47−Arg57) containing the RNA-binding domain of Tat protein (Figure 1). Dissociation constants of the Tat peptide−RNA complexes were determined from multiple sets of experiments under the same conditions used for oligocarbamate−TAR RNA complexes. These experiments showed that the Tat peptide (47−57) binds TAR RNA with a K_d of 0.78 μM. A relative dissociation constant (K_{rel}) can be determined by measuring the ratios of wild-type Tat peptide to the oligocarbamate dissociation constants (K_d) for TAR RNA. Our results demonstrate that the calculated value for K_{rel} was 0.69, indicating that the carbamate backbone structure did not significantly alter the TAR binding affinities of the unnatural biopolymer. Specificity of the oligocarbamate−TAR RNA complex formation was addressed by competition experiments.[13] The oligocarbamate−RNA complex formation was inhibited by the addition of unlabeled wild-type TAR RNA and not by a mutant TAR RNA. These results indicate that the tat-derived oligocarbamate can specifically recognize TAR RNA.

How does the tat-derived unnatural biopolymer interact with TAR RNA? Several lines of evidence suggest that Tat protein interacts with TAR RNA in a widened major groove.[11,12,14,15] Recently, we have used a site-specific cross-linking method based on 4-thiouracil (4-thioU) photochemistry to determine the conformation of TAR RNA and its interaction with Tat protein under physiological conditions.[15] To probe the oligocarbamate−RNA interactions, we synthesized TAR RNA containing 4-thioU at position 23 and performed photo-cross-linking experiments (Figure 3). Irradiation of the oligocarbamate−RNA complex yields a new band with electrophoretic mobility less than that of the RNA (lane 4). Both the oligocarbamate and UV (360 nm) irradiation are required for the formation of this cross-linked RNA−oligocarbamate complex (see lanes 3 and 4). Since the cross-linked oligocarbamate−RNA complex is stable to alkaline pH (9.5), high temperature (85 °C), and denaturing conditions (8 M urea, 2% SDS), we conclude that a covalent bond is formed between TAR RNA and the oligocarbamate during the cross-linking reaction. To test the protease stability of the oligocarbamate−RNA complexes, we subjected the oligocarbamate−RNA cross-link products to very vigorous proteinase K digestion which showed that the complexes were completely stable and there were no signs of oligocarbamate degradation

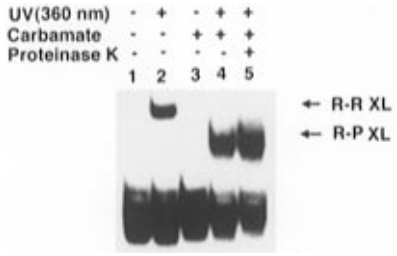

Figure 3. Site-specific photo-cross-linking reaction of TAR RNA duplex labeled with 4-thioU at position 23 with the oligocarbamate. The RNA−RNA and RNA−oligocarbamate cross-links are indicated by R−R and R−P XL, respectively. For photochemical reactions, RNA duplex was prepared by hybridizing two strands.[15] Strand 1 of the duplex was 5′-end labeled with ^{32}P. Pre-formed RNA duplexes (0.04 μM) in the absence or presence of the oligocarbamate (1.5 μM) were irradiated (360 nm) and analyzed by denaturing gels as described earlier.[15]

(lane 5). Under similar proteinase K treatment, Tat−TAR photo-cross-link products resulted in a complete loss of RNA−protein cross-link and a gain in free RNA as observed by band intensities on the gel.[15,16]

Specificity of the cross-linking reaction was established by competition experiments. Cross-linking was inhibited by the addition of unlabeled wild-type TAR RNA and not by a mutant TAR RNA lacking the trinucleotide bulge.[17] Therefore, we conclude that formation of a specific RNA−oligocarbamate complex between TAR RNA and tat-derived oligocarbamate is necessary for photo-cross-linking. As the amount of wild-type competitor RNA was increased, a decrease in RNA−oligocarbamate cross-link was observed (as expected); however, an increase in RNA−RNA cross-link was also observed. These results indicate that interstrand RNA cross-link is inhibited by the presence of RNA-binding oligocarbamate ligand. A similar result was previously obtained in photo-cross-linking experiments employing a 34 amino acid Tat fragment and 4-thioU-containing TAR RNA.[15]

These findings show that a small tat-derived oligocarbamate binds TAR RNA specifically and interacts in the widened major groove of TAR RNA. Due to the difference in backbone structure, oligocarbamates may differ from peptides in hydrogen-bonding properties, lipophilicity, stability, and conformational flexibility. Moreover, oligocarbamates are resistant to proteinase K degradation. These characteristics of oligocarbamates may be useful in improving pharmacokinetic properties relative to peptides.[6] RNA recognition by an unnatural biopolymer provides a new approach for the design of drugs which will modulate RNA−protein interactions.

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Supporting Information Available: Figures 4S and 5S (3 pages). See any current masthead page for ordering and Internet access instructions.

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