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Binding of a Cyclic BIV β-Tat Peptide with its TAR RNA Construct

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Abstract—The ability of RNA structures to adopt diverse yet complex tertiary structures has resulted in numerous fascinating RNA-protein recognition events. It was recently reported that a close relative of the HIV Rev peptide, namely a 17 residue Tat peptide from bovine immuno-deficiency virus (BIV), is able to bind to the 28 nucleotide BIV TAR RNA construct. Here we report that by simply converting the 17 residue β-ribbon peptide structure to a 19 residue cyclopeptide, the binding affinity (K_d) of the resulting cyclopeptide to the TAR RNA target, observed by fluorescence binding study, was enhanced approximately 5-fold.

RNA–protein recognition has been a very important event in many biological processes, most notably in the proper regulation of gene expression and protein translation. The progress towards understanding the paradigm of the RNA–protein recognition event has been aided immensely through the large repertoire of RNA molecular structures. Such diverse yet complex RNA structures have thus resulted in fascinating RNA–protein recognition events.[1–9] There have been numerous reports in the literature on the structure of RNA–peptide complexes, with the insertion of α-helical peptides into either the minor or major groove of double helical RNA being the most prevalent mode of interaction.[10,11] One well-studied example of such a recognition event is the interaction of the HIV rev response element (RRE) IIB RNA construct with the Rev protein (Scheme 1).[12–15] However in recent reports, a new but related system from bovine immuno-deficiency virus (BIV), has yielded an example of how a 17 residue β-sheet Tat peptide is able to bind to the 28 nucleotide (nt) BIV TAR RNA construct.[16–20] To the best of our knowledge, this is the first report in which RNA–peptide interaction involves a β-sheet peptide structure being bound to the RNA molecule.

In the BIV TAR RNA construct, there are two unpaired RNA nucleotides (Scheme 2). It is well established that these two unpaired nucleotides, within the otherwise regular RNA helix, allow the opening of the major groove enabling the insertion of a β-ribbon Tat peptide structure.[17,18] The shape of the two stranded antiparallel β-ribbons closely matches that of a double-stranded nucleic acid, which allows easy fit into a wide variety of nucleic acid structures, such as the minor grooves of both DNA and RNA, DNA major grooves, and distorted RNA major grooves.[17,18] It has also been observed that the 2′-OH groups in the RNA minor grooves are also regularly spaced so as to facilitate hydrogen bonding contacts between the backbone carbonyl groups from antiparallel sheets and the RNA.

With the structural information for the BIV Tat peptide–TAR RNA interaction, we hypothesized that 'pre-cyclizing' the BIV Tat peptide would lead not only to minimal perturbation of the overall structural integrity of the peptide, but it should also lead to higher binding affinity towards the TAR RNA construct. In this paper, we examine and compare the binding properties between the *wt* 17 residue BIV Tat peptide and an artificially constructed 19 residue cyclic BIV Tat peptide to its 28 nt BIV TAR RNA construct (Scheme 2). Understanding and developing cyclized peptides as potential antagonists toward numerous biological events has been of keen interest. First, it has been well documented that constraining the conformational freedom of the peptide would lead to a decrease in entropy of the peptide, and thus lead to higher binding affinity peptides toward the target RNA molecular construct. Recent examples of

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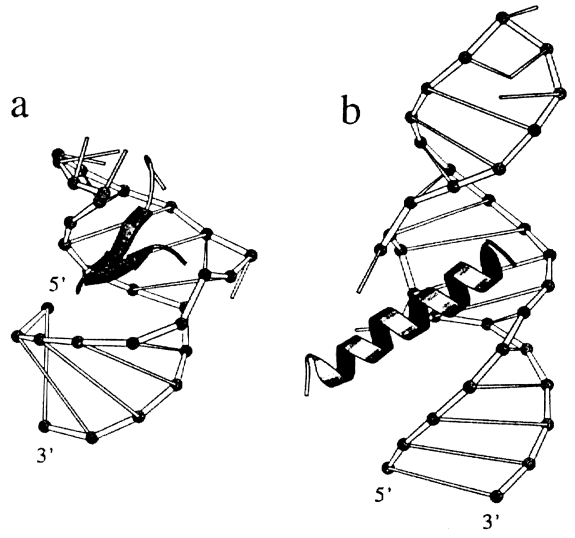


Scheme 1. NMR structures of RNA–peptide complexes:[5] (a) β-sheet BIV Tat peptide binding to its BIV TAR RNA construct; and (b) α-helical HIV Rev peptide with its HIV RRE RNA construct.

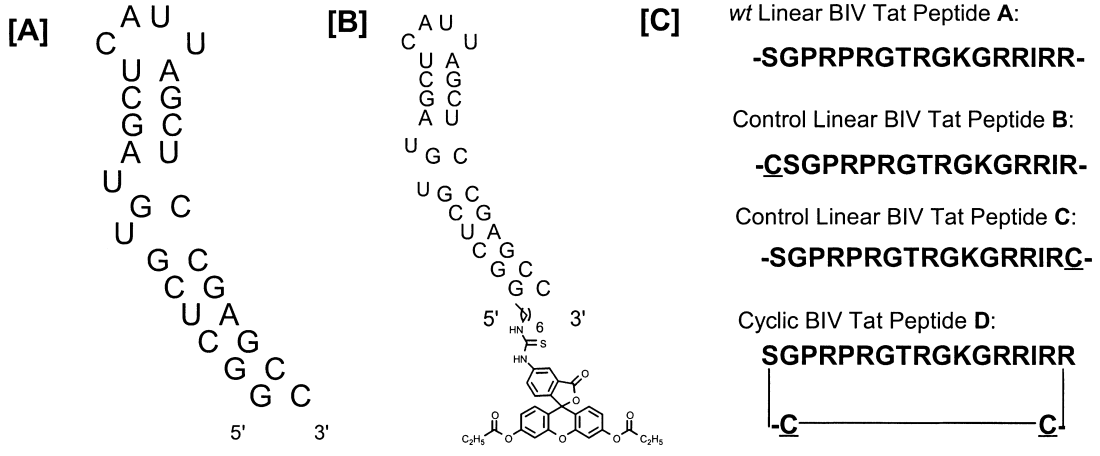
cyclic peptides toward biologically important targets, such as Streptavidin or the integrin receptor GPIIb/IIIa, have shown 3- to 5-fold enhancement in binding compared to the linear peptide.[21–23] Second, cyclic peptidic antibiotics are of immense importance as potential drug candidates, as they have been discovered to possess antiviral and antitumor activities. For example, the cyclopeptide Didemnin B displayed antineoplastic and antiviral activity in vitro, and has been shown to be a potent inhibitor of cell growth in human tumor stem-cell assays at concentrations from 1 to 100 nM.[24–26] Another recent example is the macrocyclic peptide Cycloviolins, which was reported to possess anti-HIV activity.[27]

The *wt* 17 residue BIV tat peptide A has been shown to bind to its corresponding 28 nt BIV TAR RNA construct (Scheme 2) predominantly through its basic residues.[6] It has also been reported that the BIV Tat peptide–TAR RNA binding interaction is a very tight event, with dissociation constants (K_d) in the low nM range.[6] The BIV Tat peptide–TAR RNA interaction is analogous to its close relative, namely the interaction of HIV-1 Tat peptide and its TAR RNA construct.[15]

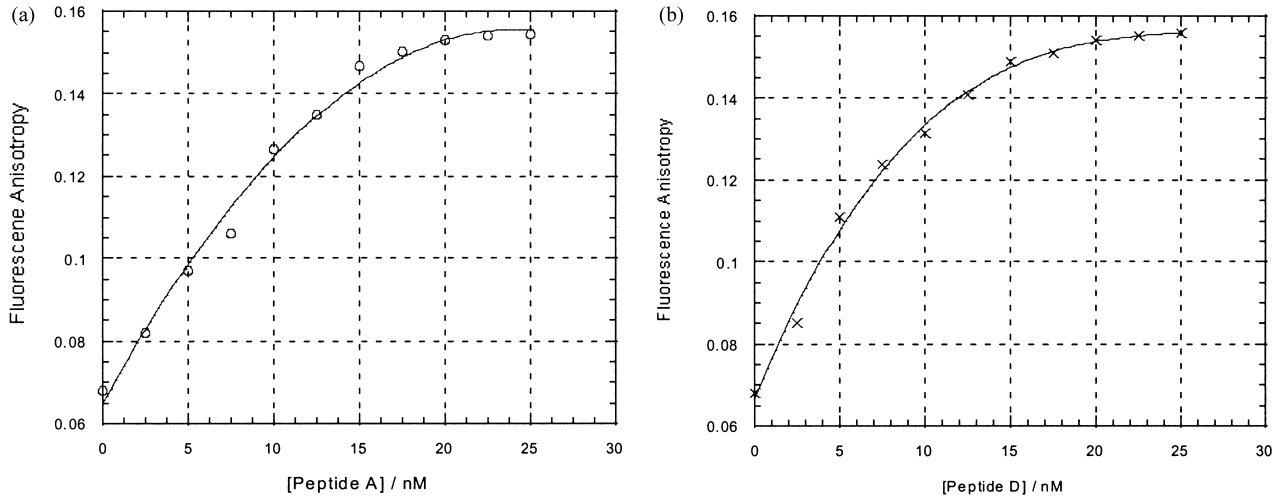
To analyze the binding events between the series of *wt* and cyclic β-sheet BIV Tat peptides with the BIV TAR RNA construct, the fluorescence binding study was employed. The fluorescence binding method is a recently developed binding-study method that enables direct and quantitative binding measurements of aminoglycoside–RNA or peptide–RNA interactions.[28,29] The basis of this methodology involves the study of either fluorescent-aminoglycoside or fluorescent-peptide conjugates with the target RNA molecules, and the monitoring of the resulting changes in fluorescence anisotropy. Here, the fluorescence binding method was slightly modified. Instead of tethering the β-sheet peptide with a fluorescence probe,[28] the target RNA construct was being labeled with the fluorescein fluorescence probe instead (Scheme 2B). This reported binding study method, in which the RNA target was labeled, has also recently been successfully utilized towards neomycin aminoglycosides.[30]

A series of β-sheet peptides **A** to **D** (shown in Scheme 2) was obtained commercially, with peptide **A** being the *wt* 17 residue Tat peptide. In addition, two control peptides **B** and **C** were constructed. These two 18 residue peptides **B** and **C** comprise a cysteine amino acid residue at either the N-terminal or C-terminal, respectively. Last, the cyclic peptide **D** was synthesized by first attaching two cysteine residues at both the N- and the C-termini, and allowing the 19 residue peptide to cyclize through disulfide bond formation.

In an incubation buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 20 mM HEPES (pH 7.40), 10 nM of the fluorescein-labeled BIV TAR RNA construct was gradually titrated with an increasing concentration of the prepared peptides. When peptide **A** was added to the fluorescein labeled TAR RNA, it was observed that the fluorescence curve gradually increased



Scheme 2. (A) The secondary structure of 28 nt BIV TAR RNA hairpin as predicted by Mfold program;[31] (B) the BIV TAR RNA construct being tethered to a fluorescein fluorescence moiety; and (C) the sequences of the linear and cyclic peptides **A** to **D** utilized in this binding study towards the BIV RNA hairpin construct (underlined residue is added on).



Scheme 3. Fluorescence titration of 10 nM of fluorescein-labeled BIV RNA construct with: (A) increasing concentration of *wt* linear BIV Tat peptide **A**; and (B) increasing concentration of cyclized peptide **D**.

and saturated at around 20–25 nM (Scheme 3a). Using a previously described curve fitting equation,[28] the K_d between BIV peptide **A** and its TAR RNA was calculated to be 1.321±0.078 nM. This K_d value is comparable to the binding results observed by Chen and Puglisi, where they have obtained a K_d of approximately 0.5 nM using the technique of gel-mobility assay.[16] Thus the K_d results obtained through utilizing fluorescence anisotropy lend evidence that the modified fluorescence binding method adopted is suitable for this investigation.

Repeating the above experimental procedure for peptides **B** to **D**, a saturation curve was again observed for each peptide, with the fluorescence curve gradually increasing and saturating at around 30 nM (the fluorescence titration curve not shown for peptides **B** and **C**). Using the previously described curve fitting equation, the K_d for all the binding events can again be calculated (Table 1). The control peptides **B** and **C** also showed a comparable K_d of 1.277±0.087 and 1.332±0.121 nM, respectively. However for cyclopeptide **D**, the K_d towards the BIV TAR RNA construct was calculated to be 0.273± 0.011 nM. The obtained K_d value between the cyclic peptide **D** and its TAR RNA was especially interesting. By simply cyclizing the linear peptide **A**, the K_d value was observed to show an improvement in binding affinity from 1.321 to 0.273 nM, which is approximately a 5-fold enhancement. Granted the linear *wt* peptide **A** has been shown to adopt a β-ribbon structure when bound to the BIV TAR RNA construct,[17,18] and does so with a very tight K_d of 1.32 nM. However, this work demonstrates that by simply cyclizing the linear peptide **A** to afford the cyclopeptide **D**, an additional 5-fold enhancement in K_d can be attained. These results suggest that by 'pre-organizing' the BIV Tat peptide into the presumably optimized conformation, it is possible to attain a further improvement in binding affinity to the BIV TAR RNA construct.

In conclusion, it is shown that cyclizing the β-sheet BIV Tat peptide, thus restricting its conformational flexibility, leads to an enhancement of the peptide's binding efficiency towards the BIV TAR RNA. Through fluorescence measurements, it was observed that an approximately 5-fold enhancement of the binding affinity of such cyclic β-peptide could be achieved compared to that of the linear conformation. These observations strongly suggest that in certain cases of peptide–RNA interactions, cyclic β-peptides can be further developed and utilized to replace their linear counterparts, as they are much more stable towards degradation by proteases and peptidases. These results will also provide us a starting premise in furthering our effort towards constructing novel cyclic peptide-based conjugated RNA binders.

Table 1. Summary of the K_ds of the various BIV Tat peptide analogues

Peptides	K_d (nM)
wt Tat BIV (**A**)	1.321±0.078
C-Tat BIV (N-terminal) (**B**)	1.277±0.087
C-Tat BIV (C-terminal) (**C**)	1.332±0.121
Cyclic Tat BIV (linked through C–C) (**D**)	0.273±0.011

Additional Proof

It was recently observed by Friedler et al. that rational design of cyclic Tat-ARM peptides are up to 4-fold more potent in inhibiting the HIV-1 Rev ARM–RRE RNA interaction than the linear Tat-ARM peptides.[32]

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