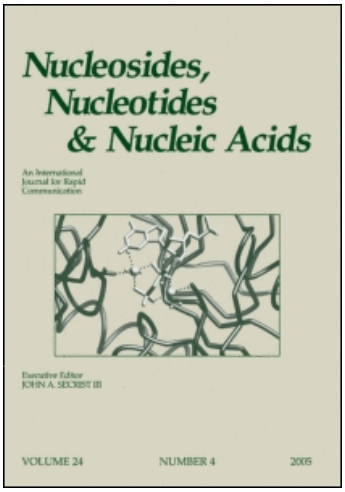

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RNA AND N3′ → P5′ KISSING APTAMERS TARGETED TO THE *TRANS*-ACTIVATION RESPONSIVE (TAR) RNA OF THE HUMAN IMMUNODEFICIENCY VIRUS-1

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ABSTRACT

We used *in vitro* selection to identify RNA aptamers able to selectively bind to the TAR RNA motif of HIV-1, an unperfect RNA hairpin involved in the transcription of the retroviral genome. We selected aptameric RNA hairpins giving rise to kissing complexes with TAR. The N3′ → P5′ phosphoramidate variant of the aptamer bind to TAR with a Kd in the low nanomolar range. However, only the RNA-RNA loop-loop complex is recognized by the Rop protein of *E. coli* which is specific for kissing complexes.

INTRODUCTION

So-called kissing complexes are formed upon the association between hairpin structures through loop-loop base-pairing. Such RNA-RNA complexes have been

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demonstrated to play a key role in various biological processes. The control of ColE1 plasmid copy number by sense-antisense RNA interaction is by far for the best characterized of these processes: RNA II, the precursor of the primer oligoribonucleotide forms a stable hybrid with the DNA template, thus generating a substrate for RNase H (1). The RNA II cleavage product then primes DNA synthesis by Pol I. The formation of the RNA II-DNA hybrid is negatively controlled by RNA I, an antisense species complementary to RNA II which therefore prevents the hybridization of RNA II to the DNA template. Both RNA I and RNA II form three stem-loops structure which participate in the RNA-RNA complex formation. The interaction between the two complementary RNA actually begins by loop-loop pairing. This transient kissing complex is then extended to generate a double-stranded RNA I-RNA II duplex along the entire length of RNA I. In addition the ColE1 replication is also controlled by a plasmid-encoded protein termed Rop (or Rom) (2). This 63 amino acids protein stabilizes the three hairpin pairs, thus promoting the extended complex formation.

Other natural RNA-RNA kissing complexes have been characterized. In particular the dimerization of retroviral genomes is also driven by loop-loop interactions. This process is thought to play a crucial role for the encapsidation of the retroviral RNA and for reverse transcription. The dimerization initiation site (DIS) adopts a hairpin structure with a palindromic loop (3, 4). This autocomplementary loop initiates RNA dimerization which then proceeds to the formation of an extended complex. Interestingly, mutations in the kissing loop of the DIS of HIV-1 which affect the dimerization reaction also reduce viral infectivity.

The biological roles played by these kissing complexes fully justify structural studies. The solution structure of a loop-loop complex related to RNA I-RNA II was solved by NMR spectroscopy (5). (Due to an increased stability the kissing formed by hairpins with $5' \rightarrow 3'$ inverted loop sequences was studied instead of the actual RNA I-RNA II complex). The structure shows that all the seven bases of the complementary loops are paired. In addition the loop-loop helix is bent toward the major groove allowing quasi-continuous stacking from one stem to the other through the loop-loop duplex. The connectors crossing the grooves are constituted by a single ribose-phosphate unit. As a consequence two phosphate residues are brought close to each other, likely providing a binding site for a magnesium ion. Preliminary structural data are available for the DIS dimer of two isolates of HIV-1 (6). Even though loop autocomplementarity is partial and consequently, in contrast to the RNA I-RNA II complex, the loop-loop interaction does not involve every loop base, the overall organization of the DIS dimer also shows a continuous stacking from one stem to the other.

Many RNA structures have been demonstrated to play a key role in gene expression, either as a signal or as a binding site for regulatory proteins. In order to interfere with these processes it is therefore of interest to design ligands exhibiting high and selective affinity for RNA structures. Besides selective complementary (7) and triplex-forming oligonucleotides (8, 9, 10) we have used an *in vitro* selection procedure to identify RNA sequences which would specifically bind to nucleic acid

structures (11, 12). It turned out that the vast majority of the RNA aptamers selected against the *trans*-activating responsive (TAR) element of HIV-1 generates kissing complexes with the TAR RNA hairpin (13). We review below the key features of these anti-TAR RNA aptamers and report some recent results on aptamer derivatives as well as on the recognition of TAR RNA-aptamer RNA kissing complexes by the Rop (Rom) protein.

MATERIALS AND METHODS

1) Oligonucleotide Synthesis

RNA molecules including the biotinylated TAR element were synthesized on an Expedite synthesizer and purified by electrophoresis on polyacrylamide/urea gels and desalted on Sephadex G25 spin columns. N3′ → P5′ phosphoramidate oligodeoxynucleotides were prepared as previously described (14).

2) In vitro Selection

It was performed from an RNA library comprising about 10 (11) oligomers with a 60 nucleotide random window, according to a previously published procedure (13). Briefly, aptamer-biotinylated TAR RNA complexes were captured by magnetic streptavidin beads, at room temperature (about 23°C) in R buffer (20 mM HEPES, pH 7.3 containing 20 mM sodium acetate, 140 mM potassium acetate and 3 mM magnesium acetate). After the 10^{th} round, the selected candidates were cloned and sequenced.

3) Surface Plasmon Resonance Kinetic Measurements

SPR experiments were performed on a BIAcore 2000 apparatus (Biacore AB, Sweden). 200–300 Resonance Units of biotinylated miniTAR was immobilized on CM5 sensorchips coated with streptavidin according to the procedure described previously (15). The sensorchip was allowed to equilibrate at 23°C in R buffer. Oligonucleotides were prepared in this buffer and injected at a flow rate of 20 μl/min.

Non linear regression analysis of single sensorgrams was used to determine the kinetic parameters assuming a pseudo-first order model according to Equations 1 and 2, for the association and dissociation phases, respectively,

$$\frac{dR}{dt} = k_{on}C(R_{\max} - R) - k_{off}R \qquad \text{(Eq. 1)}$$

$$\frac{dR}{dt} = -k_{off}R \qquad \text{(Eq. 2)}$$

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where R is the signal response, R_{max} the maximum response level, C the molar concentration of the injected oligonucleotide, k_{on} the association rate constant, and k_{off} the dissociation rate constant.

4) Preparation of Rop Protein

The protein Rop was expressed in BL21 (DE3) *E. coli* cells freshly transformed with plasmid p2R, kindly provided by B. Bishop and L. Regan (Yale University). Induction of the protein expression and purification of the protein were done essentially as described by Predki et al. (16), except that sodium phosphate was substituted for Tris-HCl during ion-exchange chromatography. Further purification was achieved with an additional step of chromatography on Q Sepharose fast flow. Fractions of the 0.2 → 0.4 M NaCl gradient containing Rop were pooled and concentrated to 1.9 mg/ml by ultrafiltration on Centriplus 3 (Amicon) centrifugation tubes.

Gel-shift experiments were performed according to Gregorian and Crothers (17). 5′ end-labelled hairpin was incubated with various amounts of the unlabelled partner and eventually, with Rop (2 μM). Incubations were done for 30 min at 4°C in TBM (Tris-borate 89 mM; $MgCl_2$ 5 mM) containing 1.5% glycerol and 0.001% bromophenol blue. Samples were then loaded on a 15% (75 : 1 crosslinking) polyacrylamide gel (20 × 20 × 0.15 cm) run at 300 V and thermostated at 4°C.

RESULTS AND DISCUSSION

The TAR RNA of HIV-1 is a regulatory element located at the very 5′ end of the retroviral genome. It is a 59 nt long region which folds in a very stable hairpin with a three nucleotide U-rich bulge near the apex of the TAR stem (18). This bulge is critical for binding the viral protein Tat which is required for efficient transcription of the HIV-1 genome. Moreover Tat forms a ternary complex with cyclin T1, a component of the Tat-associated kinase involved in the phosphorylation of the C-terminal domain of the RNA Polymerase 2 (18, 19). The formation of the TAR-Tat-cyclin T1 ternary complex requires a functional apical loop of the TAR element. Selection of RNA aptamers was performed against the full-length TAR hairpin. Binding studies were carried out with mini TAR or mini TARmut, truncated versions of TAR with or without the 3 nt pyrimidine bulge, respectively (Fig. 1).

1) Anti-TAR RNA Aptamers

The comparison of the different clones selected against the TAR RNA hairpin led to the definition of a consensus ligand organized as a stem-loop structure with a conserved octameric loop 5′GUCCCAGA (13). The six central bases of this loop sequence are complementary to the TAR loop of the Bru stain of HIV-1. The

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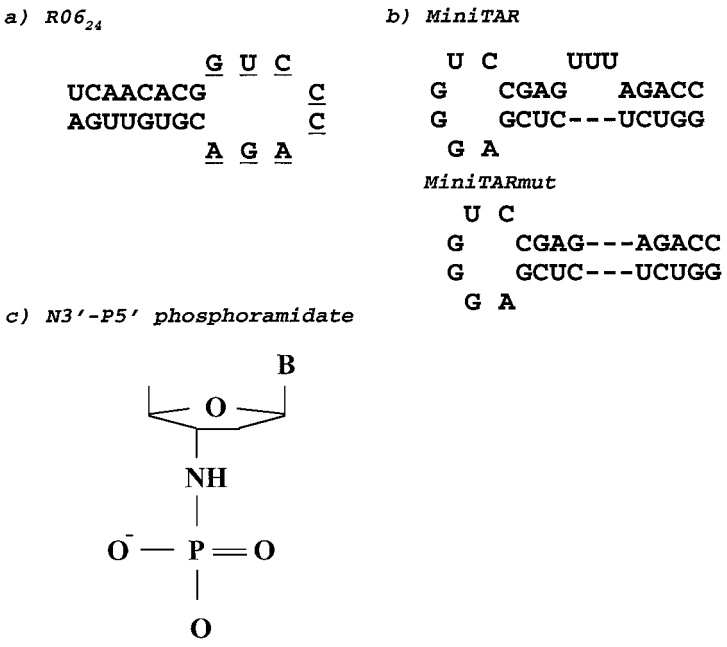

Figure 1. Sequences of the RNA aptamer (a) and of truncated TAR hairpins (b) used in this study. The N3′ → P5′ phosphoramidate nucleoside unit is also shown (c).

sequence of $R06_{24}$, a truncated version of the aptamer displaying the highest affinity for TAR is given in Figure 1.

Base-pairing between the aptamer and the TAR loops is crucial as mutations in either region abolishes or weakens the formation of the complex. Compensatory mutations may restore the interaction. Indeed, substituting the A residue by a G in the aptamer loop allowed efficient recognition of the TAR loop from the Mal strain of HIV-1 which differs from the Bru sequence by a U → C transition (13).

The conserved G and A residues closing the aptamer loop are crucial for the aptamer-TAR association (15). Any other combination but A, G drastically reduced the stability of the loop-loop complex. Interestingly, a recent selection of RNA aptamers against the yeast phenylalanine tRNA also identified kissing aptamers whose loop was closed by a G.A pair (20). This led us to ask whether this might be a signature for stable loop-loop RNA complexes. It should also be mentioned that the DIS kissing dimer involves a non-canonical closing A-A pair (21).

A hairpin secondary structure is essential to the formation of a stable aptamer-TAR RNA complex. This was not unexpected in light of previous studies between tRNA anti-codon loops and complementary sequences (22). However, it is noteworthy that the first and second base pair of the aptamer stem, next to the closing GA pair are generally 5′ purine-pyrimidine 3′ and 5′ pyrimidine-purine 3′, respectively (13). The contribution of these base pairs to the stability of the complex remain to be evaluated.

2) N3′ → *P5′* Phosphoramidate Aptamer-TAR RNA Complexes

The use of oligomers as artificial modulators of gene expression in a biological context requires in particular, resistance to enzymatic degradation. Numerous derivatives have been synthesized and evaluated. In addition to nuclease resistance, oligodeoxynucleotides containing N3′ → P5′ phosphoramidate linkages (Fig. 1) form very stable duplexes with complementary RNA strands, leading to increased melting temperatures of 2.3 to 2.6°C per modified nucleoside (14, 23). High resolution ^{1}H-NMR studies have demonstrated that the 3′-amino deoxyribose adopts predominantly a C3′-endo conformation (24). Circular dichroism studies of phosphoramidate duplexes indicated a general A-type double helix (14). X-rays analysis of crystals of the self-complementary CGCGAATTCGGG oligomer confirmed that phosphoramidate duplex adopted a RNA-like geometry (see (25) for a review). It was therefore of interest to evaluate the behaviour of an oligodeoxynucleotide N3′ → P5′ phosphoramidate derivative of the aptamer with respect to TAR binding.

We investigated by surface plasmon resonance the binding of $NP06_{24}$, the phosphoramidate analogue of $R06_{24}$, to miniTAR RNA immobilized on streptavidin-coated sensorchips. Sensorgrams obtained in R buffer at 23°C at different $NP06_{24}$ concentrations are shown in Figure 2. The curves obtained are characteristic of a pseudo-first order reaction as the dissociation phase does not show concentration dependence, in contrast to the association phase. Association and dissociation rate constants were calculated to be equal to $9.1 \times 10^4 \pm 0.02 \times 10^4\ M^{-1}s^{-1}$ and $3.7 \times 10^{-4} \pm 0.02 \times 10^4\ s^{-1}$, respectively, *i.e.* very close to the corresponding constants for the $R06_{24}$ aptamer ($k_{on} = 4.6 \times 10^4 \pm 0.02 \times 10^4\ M^{-1}s^{-1}$, $k_{off} = 2.2 \times 10^{-4} \pm 0.06 \times 10^{-4}\ s^{-1}$; not shown). This gave Kd = 4.1 nM and 4.8 nM for the

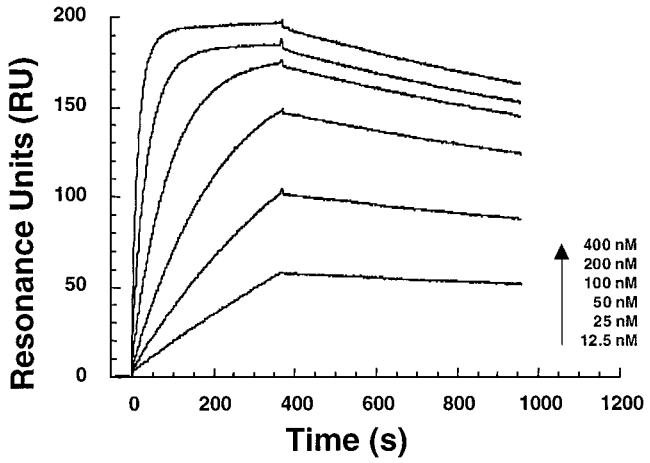

Figure 2. Sensorgrams of $NP06_{24}$-miniTAR complexes. Increasing concentrations of $NP06_{24}$ as indicated by the arrow were injected on the miniTAR-functionalized sensorchip at 23°C in R buffer. Elementary rate constants, k_{on} and k_{off}, for bimolecular complex formation were deduced from direct fitting of these curves according to Equations 1 and 2 with BIA evaluation 2.2.4 software.

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complexes of miniTAR with $NP06_{24}$ and $R06_{24}$, respectively. The stability of such complexes is highly dependent on the conformation of the molecules. Indeed, changing *a posteriori* the chemistry of a selected aptamer very generally leads to a partial loss of the desired properties. As a matter of fact the DNA derivative of $R06_{24}$ proved to be a very poor ligand of the TAR hairpin (not shown). The above results are therefore in agreement with an A-type helix of the N3′ → P5′ phosphoramidate-RNA loop-loop duplex in the $NP06_{24}$ -TAR RNA complex.

3) Recognition of Aptamer-TAR RNA Complexes by Rop

The ColE1 plasmid-encoded protein Rop is selective for loop-loop RNA helix and displays a very weak affinity for extended RNA double helix. This protein does not exhibit a strong sequence-dependence for binding and was reported to recognize the RNA I-RNA II inverted complex (17) as well as a kissing complex formed between the TAR hairpin and TAR*, a rationally designed hairpin with a loop complementary to that of TAR (26).

Band-shift assays of $R06_{24}$-TAR RNA complex demonstrate that this complex is super-shifted upon addition of Rop (Fig. 3). This further confirms that the anti-TAR aptamer does form a kissing complex with its target. In contrast, Rop did not recognize the complex formed by TAR and $NP06_{24}$, the phosphoramidate analogue of the selected sequence. Therefore, despite similar thermodynamic stability of unmodified and modified kissing TAR-aptamer complexes, the Rop protein might sense subtle conformation differences in loop-loop duplexes. Alternatively the protein might require 2′ hydroxyl groups on both strands of the loop-loop helix for binding.

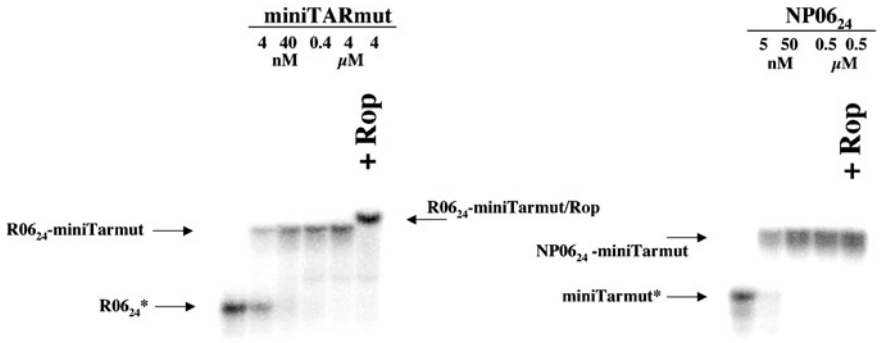

Figure 3. Band shift assay of miniTARmut-aptamer complexes. The miniTARmut hairpin was added to ^{32}P-labelled $R06_{24}$ (left) or the N3′ → P5′ phosphoramidate aptamer $NP06_{24}$ was added to the radiolabelled miniTARmut (right), at the indicated concentrations. Rop protein (2 μM) was added to the kissing complexes in the right lanes of either panel. (see materials and methods for details). Arrows indicate the labelled hairpins, the kissing and the ternary complexes, from bottom to top, respectively.

CONCLUSION

In vitro selection in RNA libraries allowed the identification of selective ligands against TAR RNA. This high affinity (Kd $\approx$ 5 nM at 23°C in the presence of 160 mM monovalent cation, 3 mM Mg^{2+}) is of interest with respect to the inhibition of biological processes in which TAR RNA is involved. The binding constant of these aptamers is about 2 orders of magnitude higher than that of a previously rationally designed hairpin loop giving rise also to kissing complexes (13). A noncanonical G-A pair closing the loop is an important parameter for stable loop-loop interactions (15).

A *posteriori* modification of our selected sequence was successful. N3′ → P5′ phosphoramidate $NP06_{24}$ were as efficient a ligand as the parent RNA molecule, likely due to a similar A-type like conformation of the loop-loop helix. Such compounds might be of interest as, in addition to their high affinity for the target, they are resistant to nuclease degradation.

The above and previously reported results (12, 13, 15) demonstrate the interest of *in vitro* selection for designing high affinity ligands of functional RNA motifs. This strategy might lead to new tools for unravelling interactions between biological macromolecules. They also extend the repertoire of oligonucleotide applications.

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