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A High-Throughput Screening Utilizing Intramolecular Fluorescence Resonance Energy Transfer for the Discovery of the Molecules that Bind HIV-1 TAR RNA Specifically

Chiharu Matsumoto, Keita Hamasaki,* Hisakazu Mihara and Akihiko Ueno

Department of Bioengineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta, Yokohama 2268501, Japan

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Abstract—A 16-residue peptide, including the $Tat_{49–57}$ sequence was labeled with a fluorescein and a tetramethylrhodamine at its N–and C-terminus, respectively. This double dye-labeled peptide was prepared as a tracer for high-throughput screening utilizing intramolecular fluorescence resonance energy transfer (FRET). The binding of the competitor molecules for HIV-1 TAR RNA were monitored and dissociation constants of those molecule were determined by using this tracer. This novel screening system might be useful to discover the drug for HIV-1 TAR RNA

The binding of transactivator protein (Tat) to the Tat responsive region of mRNA (TAR RNA) plays a key role in the replication of human immunodeficiency virus type-1 (HIV-1).[1–4] The molecule that disturbs this RNA–protein interaction might work as an anti-HIV drug. Until now, since the RNA molecule can form sequence depending intricate structure, there is no solid rule to design and construct the molecule that binds a specific RNA fragment to recognize three dimensional structure of the RNA. For the elucidation of such a rule for designing RNA-binding molecules, combinatorial chemistry would be helpful. A high-throughput screening (HTS) of the compounds might accelerate to obtain the successful results from combinatorial library. Recently, we have established and evaluated a fluorescence binding assay that enables us to monitor molecule binding with HIV-1 RRE IIB RNA using fluorescence anisotropy. Accurate dissociation constants of the molecules that bind RRE IIB RNA could be determined successfully with this system.[5–7] However fluorescence anisotropy requires the measurements of fluorescence intensity with four different combinations of polarizers. It is a mechanically time-consuming process. Single measurement of a spectroscopic signal for each sample is favorable for HTS. Furthermore, some compounds may not change their fluorescence anisotropy upon binding. It is indeed the case, as described below, that fluorescent Tat peptide does not change its fluorescence anisotropy upon binding with TAR RNA. Here, we would like to report a novel HTS utilizing fluorescence resonance energy transfer (FRET), targeting the HIV-1 trans activation responsive element of the mRNA fragment (TAR RNA) as a very useful method for the compound screening.

There is little structural information concerning Tat protein, although some structural change to the Tat peptide is expected when it forms a complex with TAR RNA. On the other hand, when the fluorescence dye, fluorescein, rhodamine or cyanine for example, forms a dimer in aqueous solution, significant fluorescence quenching occurs.[8,9] Also, fluorescein and tetramethylrhodamine is one of the nice and well characterized combinations that generate FRET when these dyes keep an appropriate distance from each other. On this basis, a 16-residue peptide including the $Tat_{49–57}$ sequence having fluorescein at its N-terminus and tetramethylrhodamine at its C-terminus (FtatRhd) has been synthesized and evaluated as tracer for HTS. We would like to present a novel fluorescence binding assay capable of applying HTS to HIV-1 TAR RNA as the target.

In order to avoid undesirable interactions between the dyes and the RNA, an additional three alanine residues were introduced into both termini of the $Tat_{49–57}$ peptide, and a cystein residue was placed on the C-terminus of the peptide for the coupling with tetramethylrhodamine maleimide. For the evaluation of

*Corresponding author. Fax: +81-45-924-5833; e-mail: khamasak@bio.titech.ac.jp

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FtatRhd, Ftat and tatRhd composed of the same amino acid sequence as FtatRhd and bearing a fluorescein and a tetramethyl-rhodamine at their N- and C-termini, respectively (Fig. 1), were prepared and compared with FtatRhd. All the peptides were synthesized by the solid phase method and purified by HPLC equipped with an ODS column, then identified by MALDI-TOFMS[10] and amino acid analysis.

When the solution of FtatRhd (100 nM) was excited at the absorption maximum of fluorescein (500 nm), it showed fluorescence emission with double peaks at 528.5 and 577 nm which derived from fluorescein and tetramethyl-rhodamine, respectively. Ftat and tatRhd has fluorescence emission peaks at 528 and 576 nm, respectively (excitation at 497 nm for Ftat and at 549 nm for tatRhd). Obviously, FtatRhd has the largest Stokes' shift (~77 nm). It is one of the advantages of FRET that it avoids scattering of excitation light. Due to the flexibility of the Tat peptide, it is possible to form an intramolecular dimer of the dyes, fluorescein and tetramethylrhodamine, and this intramolecular dimerization inactivates FRET[11] (Fig. 2A). Intrinsic fluorescence intensities of fluorescein and rhodamine in FtatRhd were 3 and 15% of Ftat and tatRhd, respectively. As the result of both intramolecular dimerization of the dyes and excited state energy transfer, fluorescence emission of FtatRhd was strongly quenched. Upon binding with TAR RNA, the intramolecular dimer of the dyes dissociates, placing distance between the two dyes and enabling the tracer to exhibit the fluorescence of FRET. As shown in Figure 2B, while the emission of FtatRhd at 528.5 nm slightly increased upon the addition of TAR RNA (wtTAR, Fig. 1), that at 577 nm increased dramatically. In the presence of an excess amount of wtTAR (1 μM),[12] fluorescence emission of FtatRhd was 2.7-fold stronger than that in the absence of the RNA. On the contrary, Ftat diminished its fluorescence upon the addition of wtTAR. This result suggests that the binding with wtTAR quenches fluorescence emission of Ftat. Even though, upon the addition of the RNA, fluorescence change of Ftat was much smaller than that of FtatRhd. In the presence of 1 μM of wtTAR, Ftat diminished its fluorescence emission by only 18%. The fluorescence anisotropy of Ftat and FtatRhd was not changed upon the addition of wtTAR. This result implies that the rotation of the dyes in the fluorescent Tat are relatively free, even in the complex with wtTAR. On the other hand, tatRhd did not change either its fluorescence intensity or fluorescence anisotropy even in the presence of 1 μM of wtTAR. These results imply that tatRhd may not bind wtTAR or, even if it binds the RNA, fluorescence of tatRhd is not affected by the binding. All these results suggest that the use of FRET is the most appropriate way to monitor the binding of Tat peptide and TAR RNA.

The dissociation constant (K_d) of FtatRhd and TAR RNA was determined by the plot of FRET emission of

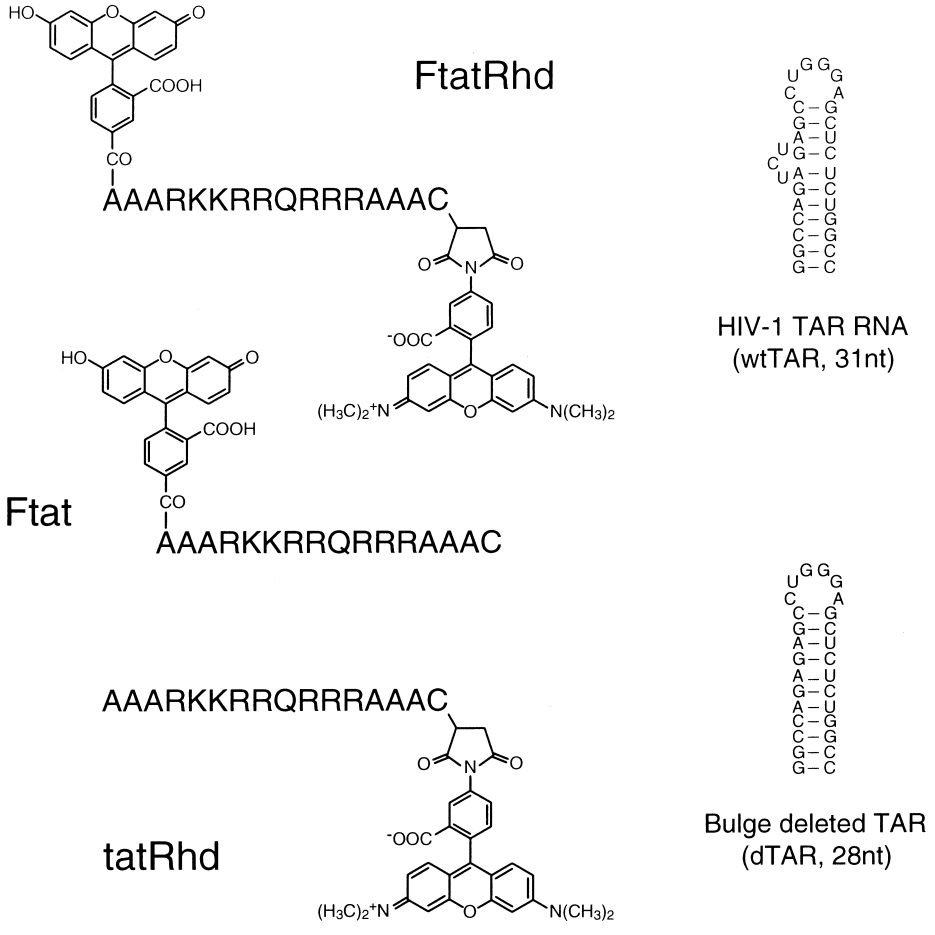

Figure 1. Dye labeled Tat peptide and secondary structure of wtTAR and dTAR.

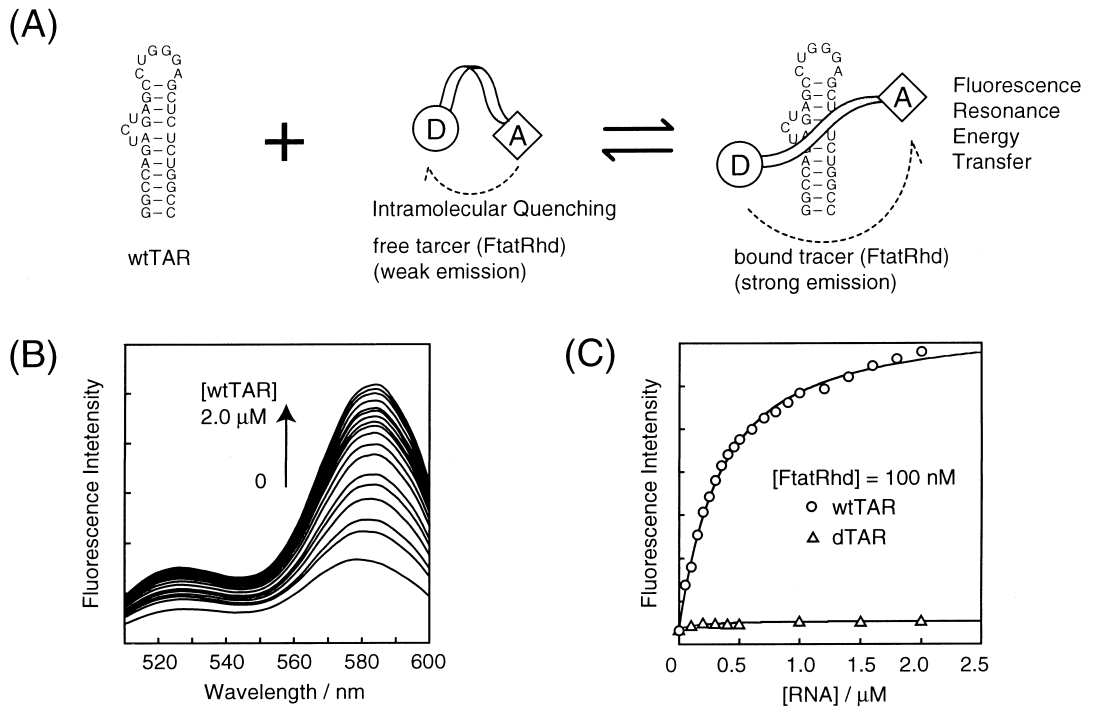

Figure 2. Tracer (FtatRhd) binding with target TAR RNA: (A) schematic illustrations of the tracer binding with target TAR RNA. The binding accompanies shape change of the tracer and enhances FRET; (B) fluorescence emission spectra of FtatRhd in the absence and presence of variable concentrations of wtTAR; (C) fluorescence intensity of the tracer (FtatRhd) as a function of the wtTAR or dTAR concentrations.

FtatRhd as a function of TAR RNA concentration (Fig. 2C) and using a least square curve fitting analysis by eq (1), with assumption of 1:1 stoichiometry.[13]

$$i = i_0 + \Delta i([\mathrm{RNA}]_0 + [\mathrm{FtatRhd}]_0 + K_\mathrm{d} - (([\mathrm{RNA}]_0 + [\mathrm{FtatRhd}]_0 + K_\mathrm{d})^2 - 4[\mathrm{RNA}]_0 \times [\mathrm{FtatRhd}]_0)^{1/2})/2 \quad (1)$$

where i and i_0 are the fluorescence intensities of the tracer in the presence and the absence of the RNA, respectively. Δi is the difference between the fluorescence intensity of FtatRhd in the presence of an infinite concentration of the RNA and in the absence of the RNA. $[\mathrm{RNA}]_0$ and $[\mathrm{FtatRhd}]_0$ are the initial concentrations of the RNA and FtatRhd, respectively. Figure 2C shows the plots of the fluorescence intensity of FtatRhd as a function of the concentration of wtTAR or bulge deleted TAR RNA (dTAR, Fig. 1). The solid curve was obtained by the analysis described above and the dissociation constant (K_d) was determined as 286 nM for wtTAR and FtatRhd. Also, this analysis revealed that 80% of FtatRhd forms a complex with wtTAR when the RNA concentration reaches 800 nM. In contrast, the additions of dTAR hardly change the fluorescence intensity of FtatRhd (Fig. 2C). It is known that a bulge structure in TAR RNA is a requirement for either Tat protein or Tat peptide binding and Tat mediated transactivation.[14–16] Therefore, the above result is one example of the evidence that FtatRhd binds wtTAR specifically.

On the other hand, when the solution of the wtTAR (800 nM) and FtatRhd (100 nM) was titrated with the wild type of Tat peptide (wt-Tat) as a competitor molecule, the fluorescence intensity of FtatRhd decreased drastically with an increasing concentration of wt-Tat peptide (Fig. 3A). This result indicates that the addition of wt-Tat peptide shifts the equilibrium of the binding toward the formation of wtTAR and wt-Tat peptide complex, that is, tracer FtatRhd in the complex was replaced with wt-Tat peptide and then free FtatRhd was released (Fig. 3B). The dissociation constant (K_D) of wt-Tat peptide was determined by the curve fitting analysis with eq (2) of 1:1 stoichiometry.[13]

$$[\mathrm{competitor}]_0 = (K_\mathrm{D}(i_\infty - i)/K_\mathrm{d}(i - i_0) + 1) \times ([\mathrm{RNA}]_0 - K_\mathrm{d}(i - i_0)/(i_\infty - i) - [\mathrm{FtatRhd}]_0(i - i_0)/(i_\infty - i_0)) \quad (2)$$

where $[\mathrm{competitor}]_0$ is the initial concentration of the competitor i_∞ is the fluorescence intensity of FtatRhd in the presence of an infinite concentration of wtTAR, and K_D is the dissociation constant of the competitor–wtTAR complex. Figure 3C shows the plot of the FRET emission of FtatRhd as a function of the concentration of the competitor, wt-Tat peptide. Then, the analysis gave 70 nM of dissociation constant for the complex of wtTAR and wt-Tat peptide.

As an evaluation of this system, the binding of the other competitor molecules, Tat mimic peptides (Table 1), in which some of either arginine or lysine were replaced by alanine residues, were prepared and their binding toward wtTAR was examined with this system. Due to the lack of some basic amino acid residues, those Tat mimics might not bind wtTAR as tightly as wt-Tat peptide. Dissociation constants for T61, which lacks

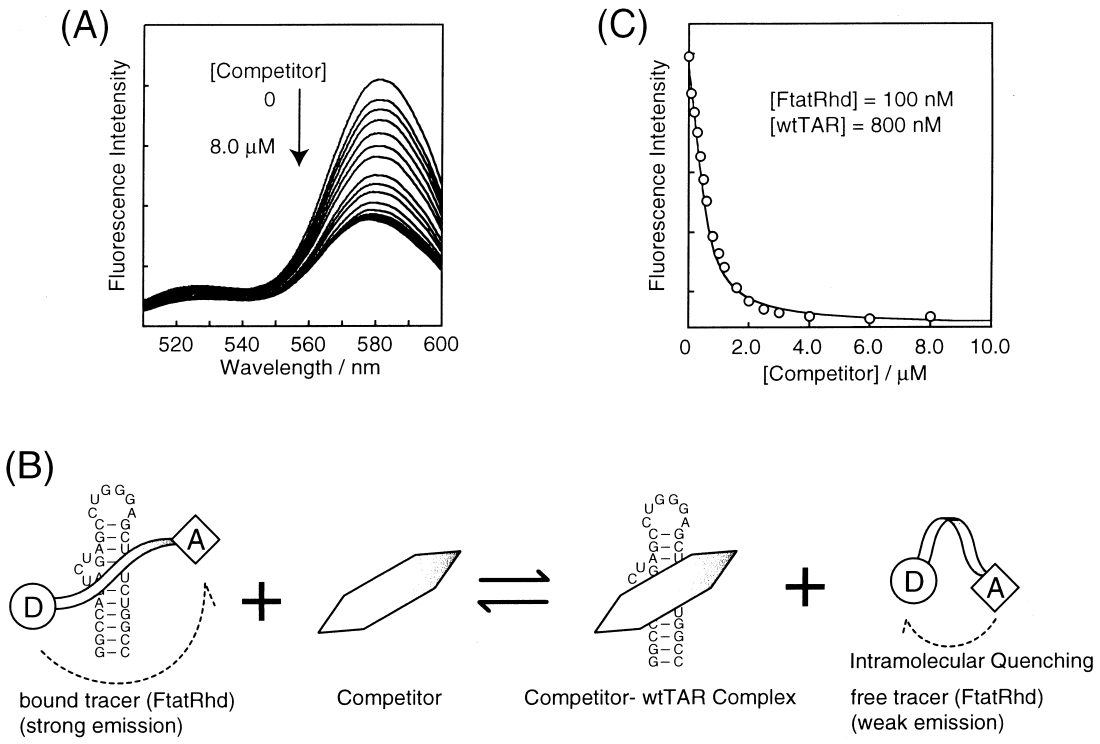

Figure 3. Competitive binding assay utilizing FRET, targeting HIV-1 TAR RNA: (A) fluorescence emission spectra in the presence of TAR RNA and variable concentration of Tat peptide as a competitor; (B) schematic illustration of the competitor binding with target HIV-1 TAR RNA; (C) fluorescence intensity of the FtatRhd (100 nM) in the presence of HIV-1 TAR RNA (800 nM) and variable concentration of Tat peptide as a competitor.

Table 1. The competitor peptide sequences and their dissociation constants with TAR RNA

Notation	Peptide sequence	K_D (nM)
wt-Tat	AAA**RKKRRQRRR**AAA	70
T81	AAA**RKKRRARRR**AAA	80
T71	AAAA**KKRRARRR**AAA	170
T61	AAA**R**AA**RR**A**RRR**AAA	2900
T51	AAAAA**RRARRR**AAAAA	18,000
T52	AAAAA**RRRRR**AAAAA	66,000
T41	AAAAA**RRARR**AAAAA	No binding
T31	AAAAA**RRAR**AAAAAA	No binding

two lysine residues of wt-Tat, and T51, which lacks an arginine and two lysine residues of wt-Tat, were 2.9 and 18 μM, respectively. These binding affinities obtained here for Tat mimics are 41 and 257 times weaker than that of wt-Tat. T41 is the peptide composed of four arginine and 12 alanine residues. T31 lacks one arginine of T41. FtatRhd did not show any fluorescence change upon the addition of either T41 or T31. These results suggest that TAR RNA does not bind either T41 or T31 and, consequently, FtatRhd does not exhibit any output upon the addition of these non-binding peptides.

This novel screening system which utilizes FRET could enable us to discriminate wt-Tat, Tat mimic peptides and non-binding peptides clearly, with large differences in fluorescence emission intensity. These results demonstrate that this system is not only useful for the determination of the accurate binding affinity of the antagonist but is also applicable to the HTS, targeting HIV-1 TAR RNA.

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