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A Tat-grafted anti-nucleic acid antibody acquires nuclear-localization property and a preference for TAR RNA

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ABSTRACT

The 3D8 single chain variable fragment (3D8 scFv) is an anti-nucleic acid antibody that can hydrolyze nucleic acids and enter the cytosol of cells without reaching the nucleus. The Tat peptide, derived from the basic region of the HIV-1 Tat protein, translocates to cell nuclei and has TAR RNA binding activity. In this study, we generated a Tat-grafted antibody ($_{H3}$Tat-3D8) by replacing complementarity-determining region 3 (CDR3) within the VH domain of the 3D8 scFv with a Tat_{48-60} peptide (GRKKRRQRRRPPQ). $_{H3}$Tat-3D8 retained the DNA-binding and DNA-hydrolyzing activity of the scFv, and translocated to the nuclei of HeLa cells and preferentially recognized TAR RNA. Thus, the properties associated with the Tat peptide were transferred to the antibody via Tat-grafting without loss of the intrinsic DNA-binding and hydrolyzing activities of the 3D8 scFv antibody.

1. Introduction

The HIV-1 Tat protein comprises 86 amino acid residues (aa) and plays an essential role in HIV-1 replication by specifically interacting with the *trans*-activation responsive region (TAR) RNA. TAR RNA is a 59-base pair stem-loop structure located at the 5′end of all HIV mRNAs [1–3]. Functional studies for domains within the HIV Tat protein have shown that the peptide of residues 49–57 (RKKRRQRRR), derived from the highly basic region of the HIV Tat protein, binds to TAR RNA with high affinity [4–6], penetrates cell membrane, and accumulates in the nucleus [7,8]. Because of its cell-penetrating and/or nuclear localizing ability, the basic Tat peptide has been used for the intracellular delivery of various cargos, including proteins, nanoparticles, chemicals and nucleic acids. Tat peptides of 9–13 aa length, such as Tat_{49-57}, Tat_{48-57}, Tat_{47-57}, Tat_{48-58}, Tat_{47-58} or Tat_{48-60} have all been used in different experimental settings [9,10]. However, most studies of Tat-fused molecules have focused on cellular entry and cellular localization, with no interest in its TAR RNA binding activity. A recent study showed that RNase T1-Tat, comprising the Tat_{49-57} peptide grafted into peripheral loop 3 (L3) of RNase T1, maintains RNA-hydrolytic activity and continues to target TAR RNA [11].

We previously showed that the 3D8 single chain variable fragment (3D8 scFv) nonspecifically binds to and hydrolyzes nucleic acids [12–14]. Furthermore, the single variable domains within the heavy and light chains (3D8 VH and VL) efficiently bind and hydrolyze DNA. However, the relative DNA binding affinities of 3D8 VH and VL do not correlate with their catalytic activities; VH has ~10-fold higher DNA binding activity than VL, but the DNA hydrolyzing activity of VH is much lower than that of VL [12]. Furthermore, the 3D8 scFv protein enters cells via caveolae-mediated endocytosis, after which it localizes within the cytosol without translocating to the nucleus [13]. This led us to speculate that grafting the HIV-1 Tat peptide into the complimentarity-determining region (CDR) within the VH domain of the 3D8 scFv may enable nuclear localization and high affinity binding to TAR RNA without destroying intrinsic DNA binding and hydrolyzing activity of the antibody. To the best of our knowledge, there are no previous reports of Tat-grafting into the CDR of an antibody (Ab).

In the present study, we designed a Tat-grafted 3D8 scFv (designated $_{H3}$Tat-3D8), in which the VH CDR3 within the 3D8 scFv was replaced with a Tat_{48-60} peptide (GRKKRRQRRRPPQ). We obtained a soluble form of the $_{H3}$Tat-3D8 protein from bacterial cultures that could translocate into the nucleus and bind preferentially to the viral TAR RNA stem-loop structure, while retaining the original DNA hydrolyzing activity of 3D8 scFv. Our results suggest that the properties of a functional peptide can be transferred to an Ab by grafting the peptide into its CDR.

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2. Materials and methods

2.1. Construction of $_{H3}$Tat-3D8

The Tat-grafted 3D8 VH gene (~350 bp), in which the CDR3 of the 3D8 VH domain was replaced with a nucleotide sequence encoding the Tat_{48-60} peptide (GRKKRRQRRRPPQ), was synthesized by Integrated DNA Technologies (IDT). The Tat-grafted 3D8 VH gene was subcloned into the *Xma*I/*Xba*I sites of the pIg20-3D8 scFv expression vector [12], which contains a Pho A leader sequence at the N-terminus and a Staphylococcal protein A tag at the C-terminus, to generate pIg20-$_{H3}$Tat-3D8.

2.2. Expression and purification of proteins

The $_{H3}$Tat-3D8, 3D8 scFv, and HW1 (control) scFv proteins [15] were expressed and purified from bacterial culture supernatants using IgG-Sepharose affinity chromatography as previously described [12]. Protein concentrations were determined using the unique extinction coefficients of each protein, which were calculated from the amino acid sequence [16].

2.3. Enzyme-linked immunosorbent assay (ELISA)

Purified proteins (5 μg/well) were incubated in wells coated with pEGFP-N2 DNA plasmid DNA (5 μg/ml) (Clontech). 3D8 scFv bound to the DNA was detected using rabbit anti-3D8 scFv Ab and an alkaline phosphatase-conjugated anti-rabbit secondary Ab, as previously described [12].

2.4. DNA-hydrolyzing assays

The DNA hydrolyzing assay was performed as described previously [12]. Briefly, supercoiled pEGFP-N2 (200 ng; used as the substrate) was incubated with different Abs (800 nM) for 1 h at 37 °C in TBS buffer, pH 7.5, containing 2 mM $MgCl_2$. The reaction mixtures were then analyzed by electrophoresis on 1% agarose gels. For the *in situ* DNA hydrolysis assay [15,17], 5 μg of the purified Abs were applied to a set of 12% SDS-PAGE gels containing 20 μg/ml pEGFP-N2 DNA. The SDS was removed by incubating the gels with 7 M urea for 1 h at room temperature (RT). After washing three times with buffer A [(10 mM Tris, pH 7.4, containing 10 mM $MgCl_2$, 50 mM NaCl, and 0.1% (v/v) TritonX-100)], the gels were incubated in buffer A for 12 h at 37 °C to allow renaturation of the proteins. The gels were then stained with ethidium bromide to visualize the regions of DNA-hydrolysis. Parallel longitudinal gel slices were also stained with Coomassie Blue to estimate the molecular masses of the proteins.

2.5. Immunofluorescence assays

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Flow cytometry and confocal microscopy and were then used to detect intracellular proteins [13]. For flow cytometry, cells (1×10^5 cells/well) were incubated with each of the proteins (5 μM) or FITC-conjugated Tat_{48-60} peptide (5 μM) for 6 h at 37 °C. Cells were harvested, resuspended, and then once again treated with 0.1% trypsin for 3 min at 37 °C to remove any surface-bound proteins. The cells were then washed once with cold PBS buffer (50 mM sodium phosphate, 100 mM NaCl, pH 7.2) containing 2% FCS, and fixed with 2% paraformaldehyde in PBS for 1 h at 4 °C. Cells were permeabilized with Permbuffer (1% BSA, 0.1% saponin, 0.1% sodium azide in PBS) for 10 min at RT, and incubated with a polyclonal rabbit anti-3D8 scFv Ab followed by FITC-anti-rabbit IgG (Sigma) and analyzed (1×10^4 cells per tube) using a FACSCalibur flow cytometer (Becton Dickinson). For confocal microscopy, cells grown on glass cover slips in 24-well plates at a density of 5×10^4 cells/well were incubated for 6 h at 37 °C in the presence of proteins (5 μM) or FITC-Tat_{48-60} (5 μM). Cells were then washed, fixed, and processed as described above. The proteins were detected using a rabbit anti-3D8 scFv Ab followed by a TRITC-anti-rabbit IgG secondary Ab (Sigma). Finally, the nuclei were stained with DAPI for 10 min at RT. Images were obtained using a laser scanning confocal fluorescence microscope (model LSM510, Carl Zeiss).

2.6. Affinity measurement

The affinity of the proteins for the synthesized nucleotides was assessed using a Biacore 2000 SPR (Surface Plasmon Resonance) biosensor (Amersham Biosciences) [12]. Biotinylated-TAR RNA and a random-sequence single-stranded (ss) RNA (N57) were synthesized by Integrated DNA Technologies (IDT). Briefly, biotinylated-TAR RNA, or N57, was immobilized on a streptavidin-coated sensor chip. Proteins (5–200 nM), prepared by serial dilution in HES, were injected into the flow cell at a rate of 50 μl/min for 3 min, followed by a constant flow of HES buffer at 50 μl/min for 3 min. Bound proteins were removed by injection of 50 mM NaOH/1 M NaCl to regenerate the chip. Kinetic parameters were determined using a nonlinear regression analysis model according to a 1:1 binding model using BIAevaluation version 3.2 software (Amersham Biosciences). The dissociation constant (K_D) was calculated using the formula: $K_D = k_{off}/k_{on}$, where k_{off} and k_{on} are the dissociation and association rate constants, respectively.

3. Results and discussion

3.1. Design of $_{H3}$Tat-3D8

$_{H3}$Tat-3D8, in which the VH-CDR3 of 3D8 scFv was replaced with the HIV-1 Tat_{48-60} peptide, is shown in Fig. 1A. Sequence and secondary structure of HIV-1 TAR RNA to which Tat_{48-60} peptide specifically binds is shown in Fig. 1B. The CDR3 in the VH domain was chosen for Tat_{48-60}-grafting because the VH domain has ~10-fold higher affinity (2.4–8.4 μM) for certain synthetic oligonucleotides than for the VL domain (3.2–72 μM), although its affinity is still lower than that of the scFv (17–74 nM). However, a kinetic study showed that VH has lower hydrolyzing activity on supercoiled plasmids than VL which has DNA hydrolyzing activity comparable with that of the scFv [12]. Thus, we anticipated that that DNA hydrolyzing activity of the VL domain would be maintained, even though the structure of the VH domain had been altered. Furthermore, CDR3 was chosen because it contains a similar number of aa residues (11 aa) to the Tat_{48-60} peptide (13 aa). The VH CDR1 and VH CDR2 domains contain only 5 and 6 aa, respectively (Fig. 1C). Grafting of Tat_{48-60} into the VH CDR3 resulted in the replacement of 9 (GAYKRGYAM) out of 11 aa (GAYKRGYAMDY). The two residues D and Y were maintained, since they are structurally involved in beta-strand formation rather than loop formation [12].

3.2. Expression and purification of $_{H3}$Tat-3D8

The soluble form of $_{H3}$Tat-3D8 was expressed in *Escherichia coli* and purified from the bacterial culture supernatant (purity >95%; Fig. 2C) with a yield of 0.5–1 mg/L. The fact that $_{H3}$Tat-3D8 was expressed in soluble form is itself noteworthy, because the soluble expression of Tat peptide-containing proteins (regardless of the position of the Tat peptide within the protein structure) has not

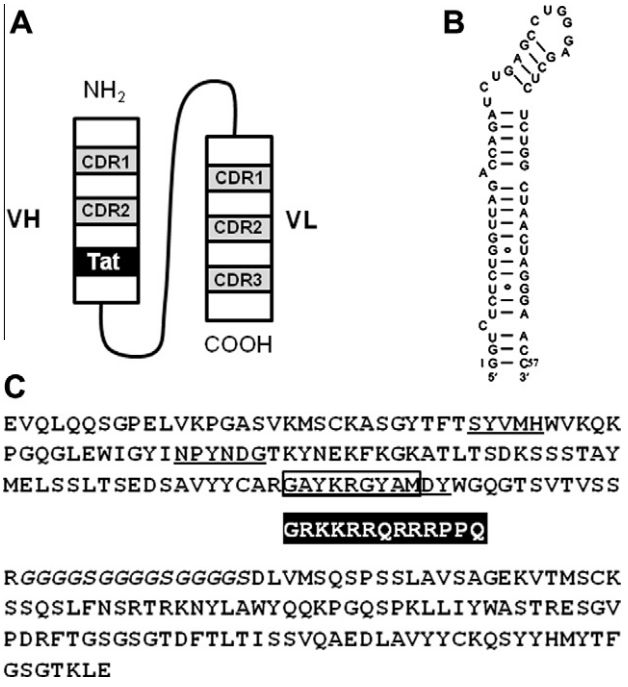

Fig. 1. Structure of $_{H3}$Tat-3D8 protein and TAR RNA. (A) Diagram showing the $_{H3}$Tat-3D8 structure. Gray and black squares indicate the CDRs and the Tat-grafted VH CDR3, respectively. (B) Sequence and secondary structure of HIV-1 TAR RNA construct. (C) Amino acid sequences of the VH and VL domains of the 3D8 scFv. The CDR residues within the VH domain are underlined. Open boxes indicate the residues within the VH CDR3 domain that were replaced with the Tat peptide (closed box).

yet been reported. Tat-fusion proteins have been purified from bacterial cell lysates only in insoluble form using urea- or guanidinium-denaturing protein purification methods, which require subsequent protein refolding [18–21]. However, we were unable to express soluble Tat-3D8 scFv proteins in which $Tat_{48–60}$ was fused to the N-terminus or grafted into VH CDR1 or CDR2 of 3D8 scFv using the same pIg20 expression vector (data not shown). This suggests that $Tat_{48–60}$ does not impair protein solubility when grafted into VH CDR3.

3.3. DNA binding and hydrolyzing activity of $_{H3}$Tat-3D8

The ELISA results showed that, like 3D8, $_{H3}$Tat-3D8 bound supercoiled plasmid DNA. No DNA binding activity was observed for HW1 scFv (control) or with PBS buffer alone, confirming that $_{H3}$Tat-3D8 retained the DNA binding activity of the parent scFv (Fig. 2A). Next we investigated whether $_{H3}$Tat-3D8 retained DNase activity. The supercoiled pEGFP-N2 plasmid was incubated with various proteins at 37 °C for 1 h and the reaction mixtures were resolved by agarose gel electrophoresis. The gels were then stained with ethidium bromide. As shown in Fig. 2B, $_{H3}$Tat-3D8 (800 nM) effectively hydrolyzed the substrate DNA after 1 h (indicated by DNA-smearing in the gels and a decrease in the quantity of supercoiled plasmid DNA). Thus, $_{H3}$Tat-3D8 appears to have a similar level of catalytic activity to that of 3D8 scFv on supercoiled plasmid DNA. To exclude the possibility of false-positives caused by contaminating nuclease activity, *in situ* DNA hydrolysis by $_{H3}$Tat-3D8 was assessed in SDS-PAGE gels containing pEGFP-N2 DNA as a substrate. Fig. 2C shows that DNA hydrolysis was observed at gel positions corresponding to the molecular mass (35 kDa) of $_{H3}$Tat-3D8, confirming that the DNA hydrolyzing activity of $_{H3}$Tat-3D8 was an intrinsic property of this protein and was not due to bacterial nucleases that could have co-purified with $_{H3}$Tat-3D8.

3.4. Nuclear translocation of $_{H3}$Tat-3D8

We next investigated the ability of $_{H3}$Tat-3D8 to invade HeLa cells. HeLa cells have been used extensively as a model cell line to investigate cell-penetrating peptides and anti-DNA Abs. Cells were incubated with proteins or $Tat_{48–60}$ for 6 h at 37 °C, prior to immunofluorescence labeling. Confocal microscopic analysis of cells treated with 3D8 scFv or $_{H3}$Tat-3D8 showed that fluorescence was localized in discrete compartments within the cytosol, suggesting internalization via an endocytic process [7,13]. $_{H3}$Tat-3D8 translocated to the nuclei of the cells, where it accumulated in a region corresponding to the nucleolus. The 3D8 scFv localized to the cytosol, with no evidence of nuclear localization, while the $Tat_{48–60}$ peptide was diffusely scattered throughout the cytosol and nucleus, consistent with previous reports [13,22,23]. No cell-penetrating activity was observed for negative control HW1 scFv (Fig. 3A). The amount of protein taken up by the cells was quantified by flow cytometry. The results indicated that

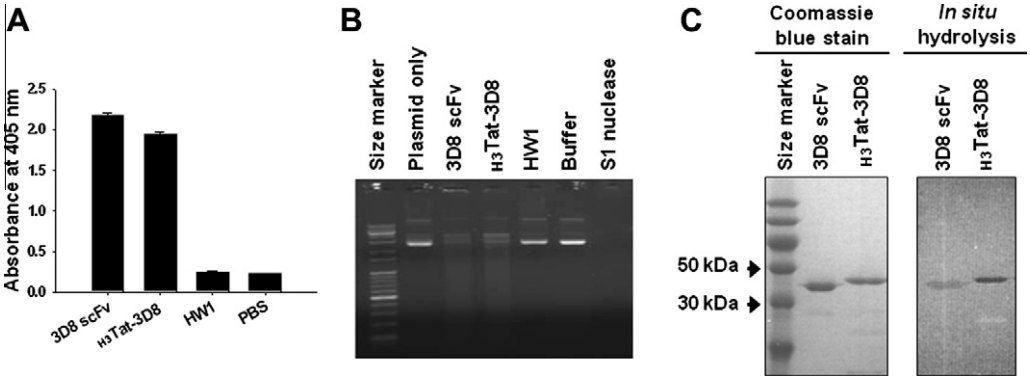

Fig. 2. $_{H3}$Tat-3D8 binds and hydrolyzes DNA. (A) ELISA. 3D8 scFv, $_{H3}$Tat-3D8, and an irrelevant HW1 control protein (0.5 μg/well) were incubated with the pEGFP-N2 plasmid DNA. The binding of the proteins to the DNA was detected using an rabbit anti-3D8 Ab. (B) The DNA hydrolyzing assay using 3D8 scFv and $_{H3}$Tat-3D8. Supercoiled pEGFP-N2 (200 ng), used as a substrate, was incubated with 3D8 scFv or $_{H3}$Tat-3D8 (800 nM) for 1 h at 37 °C in TBS buffer (pH 7.5) containing 2 mM $MgCl_2$. The reaction mixtures were analyzed by electrophoresis on agarose gels (1%) and stained with ethidium bromide. S1 nuclease was used as a positive control for hydrolysis. (C) *In situ* gel assay for DNase activity of the proteins against pEGFP-N2 DNA. Proteins (5 μg) were resolved on SDS-PAGE gels containing pEGFP-N2 (20 μg/ml). After renaturation, the gels were stained with Coomassie Blue (left panel) or ethidium bromide (right panel). DNase activity was indicated by a sharp dark band on a fluorescent background after ethidium bromide staining.

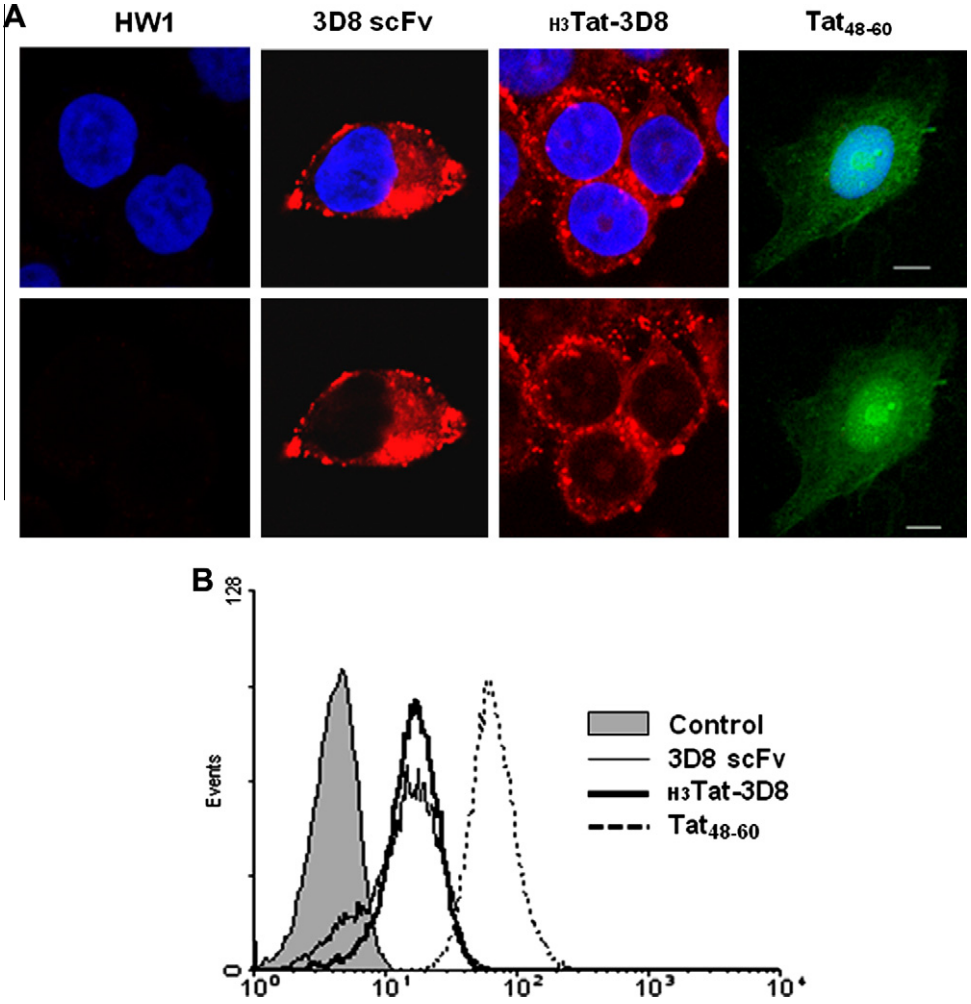

Fig. 3. $_{H3}$Tat-3D8 enters the cells and translocates to the nuclei. HeLa cells were incubated with 5 μM proteins or FITC-Tat for 6 h at 37 °C, followed by fixation and permeabilization as described in Section 2. (A) Confocal microscopy. Intracellular 3D8 scFv and $_{H3}$Tat-3D8 were detected using a rabbit anti-3D8 scFv Ab followed by a TRITC-anti-rabbit IgG secondary Ab. Scale bar, 10 μm. (B) Flow cytometry to detect endocytosis of proteins. Internalized 3D8 scFv and $_{H3}$Tat-3D8 were detected using a rabbit anti-3D8 scFv Ab followed by a FITC-anti-rabbit IgG secondary Ab.

Table 1
SPR-derived[a] kinetic binding parameters for the interaction between 3D8 scFv and $_{H3}$Tat-3D8 Abs using N57 single stranded RNA or TAR RNA as substrates.

Substrate	Proteins	K_{on} (M^{-1} S^{-1})	K_{off} (S^{-1})	K_D (M)
N57	3D8 scFv	$1.89 \pm 0.03 \times 10^5$	$2.75 \pm 0.18 \times 10^{-3}$	$1.45 \pm 0.17 \times 10^{-8}$
	$_{H3}$Tat-3D8	$2.23 \pm 0.08 \times 10^4$	$4.84 \pm 0.48 \times 10^{-3}$	$2.17 \pm 0.42 \times 10^{-7}$
TAR RNA	3D8 scFv	$2.32 \pm 0.06 \times 10^5$	$6.68 \pm 0.32 \times 10^{-3}$	$2.89 \pm 0.29 \times 10^{-8}$
	$_{H3}$Tat-3D8	$3.25 \pm 0.17 \times 10^5$	$2.37 \pm 0.06 \times 10^{-3}$	$7.29 \pm 0.08 \times 10^{-8}$

[a] At least five data sets were analyzed using different protein concentrations to determine the K_D values.

internalization of the 3D8 scFv and $_{H3}$Tat-3D8 was almost the same, with the control Tat_{48-60} showing remarkably uptake high efficiency (Fig. 3B).

Internalization of 3D8 scFv is mediated by caveolar endocytosis [13]. Two different endocytosis mechanisms have been described for the Tat peptide depending on the cargo being carried; GST-Tat_{48-58}-EGFP is internalized via caveolin-mediated endocytosis [7,24], whereas Tat-Cre fusion proteins are internalized via macropinocytosis [25]. In the present study, we did not attempt to study endocytosis as a mechanism for $_{H3}$Tat-3D8 uptake. The Tat peptide, when attached to proteins, enables them to enter cells [7,9–11]. However, not all peptide-conjugated proteins are able to translocate to the nucleus after cell entry [19,25]. This may be due to (1) an inadequate Tat peptide sequence within the fusion protein, resulting in a failure to access some elements of the nuclear transport system, or (2) to unfavorable alterations in intracellular trafficking, resulting in the inability to escape from the endocytic compartment. It should be noted, however, that $_{H3}$Tat-3D8 was able to translocate to the nucleus; possibly because $_{H3}$Tat-3D8 has a favorable structure for nuclear translocation.

3.5. $_{H3}$Tat-3D8 exhibits a preference for TAR RNA

The affinity of both $_{H3}$Tat-3D8 and 3D8 scFv for TAR RNA, which has an unique secondary structure formed by a 57 bp nucleotide (Fig. 1B), was evaluated by SPR. Proteins were allowed to flow over immobilized TAR RNA and a random-sequence 57 bp ssRNA (N57). As seen in Table 1, $_{H3}$Tat-3D8 exhibited about 3-fold higher binding

affinity for TAR (K_D = 73 nM) than for N57 (K_D = 220 nM), whereas the 3D8 scFv bound to N57 (K_D = 15 nM) with a 2-fold higher binding affinity than to TAR RNA (K_D = 30 nM). BSA (100 μM) was used as a negative control, and did not bind to any of the DNA-immobilized surfaces (data not shown). This result suggests that the preferential binding of $_{H3}$Tat-3D8 to TAR RNA was derived from the Tat_{48-60} grafted onto 3D8 scFv.

Using acrylamide gel electrophoresis of Tat-TAR complexes, the affinities of different Tat proteins for TAR RNA have been estimated to be: K_D = 12 nM for the full length Tat protein [26], K_D = 0.1 nM for Tat_{38} (Tat_{49-86}) [2], and K_D = 16 pM for Tat_{24} (Tat_{49-72}) [27]. However, the affinity of Tat-fusion proteins (either Tat-conjugated proteins or Tat-grafted proteins) for TAR RNA has not yet been quantitatively measured. RNase T-Tat, a Tat-grafted protein in which peripheral loop 3 of RNase T was replaced with a Tat_{49-57} peptide, preferentially recognizes and hydrolyzes TAR RNA, indicating the usefulness of RNase T-Tat for disrupting the initial transcription process of the HIV-1 virus [11]. Based on the results of the present study, we suggest that $_{H3}$Tat-3D8 may be used as a tool in investigations of the inhibition of HIV-1 replication.

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