BMB
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Examination of specific binding activity of aptamer RNAs to the HIV-NC by using a cell-based *in vivo* assay for protein-RNA interaction

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The nucleocapsid (NC) protein of the Human Immunodeficiency Virus-1 plays a key role in viral genomic packaging by specifically recognizing the Psi(Ψ) RNA sequence within the HIV-1 genome RNA. Recently, a novel cell-based assay was developed to probe the specific interactions *in vivo* between the NC and Ψ-RNA using *E.coli* cells (*J. Virol.* 81: 6151-55, 2007). In order to examine the extendibility of this cell-based assay to RNAs other than Ψ-RNA, this study tested the RNA aptamers isolated *in vitro* using the SELEX method, but whose specific binding ability to NC in a living cellular environment has not been established. The results demonstrate for the first time that each of those aptamer RNAs can bind specifically to NC in a NC zinc finger motif dependent manner within the cell. This confirms that the cell-based assay developed for NC-Ψ interaction can be further extended and applied to NC-binding RNAs other than Ψ-RNA. [BMB reports 2008; 41(7): 511-515]

INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) nucleocapsid (NC) protein plays important roles in several steps of the virus life cycle, particularly viral particle assembly (1, 2). It is produced through proteolytic processing of the Gag polyprotein precursor and contains two zinc finger motifs (Cys-X2-Cys-X4-His-X4-Cys), which are highly conserved among all retrovirus families (3-5). Mutations of the zinc finger domains cause major defects in the specific RNA binding activity and encapsidation of viral genomic RNA (3-10). The NC protein specifically recognizes so-called Psi (Ψ)-RNA sequences, which are approximately 120 bases long and contain four stem loops in the 5'-long terminal repeat (5'-LTR) region of the HIV-1 RNA that enable it to encapsulate selectively the viral genomic RNA among many different cellular RNAs (4, 11-13). Because the interaction is essential for the efficient packaging of the genomic RNA into a new HIV-1 virus particle, it has had bee studied extensively in an attempt to understand molecular mechanism of the specific protein-RNA interaction, such as what stem loops of the Ψ are important for binding to the NC protein and how small a portion of the nucleic acids is needed (14,15). This specific protein-RNA interaction is one of the important model systems in the field of protein-RNA interaction.

In order to further understand the specific RNA structural moiety recognized by the NC protein, many attempts have been made to search for RNA aptamers that are specific to the Gag or NC protein. Such RNA aptamers were selected from random RNA pools by repeated rounds of a selection technique *in vitro*, specifically what is known as SELEX (Systemic Evolution of Legends by Exponential enrichment) (16, 17). A number of *in vitro* selected RNA aptamer molecules obtained using this technique have been reported to bind to the HIV-1 Gag protein with high affinity (18) or to NC *in vitro* (4, 19-21). However, it is unclear if these RNA aptamers operate and bind to the NC protein with the same specificity and affinity within living cells.

A novel cell-based assay was recently developed to probe the specific interaction between the NC protein and wild type Ψ-RNA *in vivo* using *E.coli* cells (22). The aim of this study was to examine these NC aptamer RNAs in a cell-based assay not only to test the specific binding activity of the aptamer RNAs *in vivo* but also to determine the applicability and expandability of the assay to examine the NC-RNA interactions. From this study, it was found that the RNA aptamers investigated here showed specific binding activity to NC *in vivo*. This shows that the cell-based NC-Ψ interaction assay can be used effectively to examine the interactions between NC and NC specific RNAs. In addition, it was found that not all the RNA aptamers showed higher binding affinity than Ψ-RNA, which is in contrast to that reported in the *in vitro* test. This suggests that they may not operate equally under *in vivo* conditions. This highlights the need to confirm *in vivo* the binding ability of RNA aptamers isolated *in vitro* using a secondary cell-based assay, as demonstrated in this report.

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Received 31 March 2008, Accepted 8 April 2008

Keywords: Cell-based assay, HIV nucleocapsid protein, Protein-RNA interaction, Psi (Ψ), RNA aptamer

RESULTS AND DISCUSSION

In order to examine the extendibility and applicability of the cell-based assay to RNAs other than Ψ-RNA, which has been established to probe the specific interaction between NC and Psi(Ψ)-RNA via NC-Psi(Ψ) mediated Translation Repression of reporter gene (NPTR assay) in living cells (22), this study examined the RNA aptamers isolated using the SELEX method, which binds the HIV-1 NC protein with high affinity *in vitro* (21) but whose specific binding ability to NC in a living cellular environment is unclear.

The RNA aptamers were composed of a stretch of nucleotide sequences shorter than Ψ-RNA (Fig. 1A) but contained stem loop structures, such as Ψ-RNA, as previously noted (21). RNA aptamer reporter vectors containing the RNA aptamer sequences in place of the Ψ sequence in the upstream of LacZ reporter gene in the pMV/AS-Psi(Ψ)/LacZ plasmid were constructed in order to measure the *in vivo* NC binding affinity of each of the RNA aptamers (22). Each of the resulting RNA aptamer reporter vectors (pMV/AS-SE8-6/LacZ, pMV/AS-SE8-13/LacZ and pMV/AS-SE90-20/LacZ) was transformed into *E.coli*

A

Psi(Ψ)	121bp	AGGACTCGGCTTGCTGAAGCGCGCACAGCAAGAGGCGAGGGGCGGCGACTGGTGAGTACGC CAATTTTTGACTAGCGGAGGCTAGAAGGAGAGAGAGATGGGTGCGAGAGCGTCGGTATTA
SE8-6	70bp	CGCGGAAGTACGACTGGGTACTTTCCGGTAGCCGGTAGGAGTAGTCCGGGCCTCGCTTCCCG ATATCCGT
SE8-13	62bp	TATAGTGTGTTCAACGCTTAGATTGAGCCGGATTAAGGGTGTACTTTTGTCCGTTGTACGAG
SE90-20	50bp	AATTGCAGTACGTAAAGTATTGAGTCGTGGCATTGGGACATGCGCTATGG
SW8.4	45bp	TTCGTCTACAGGGAACCGTGGTGCATCTGTGAAGTTGTAGACGAA

B

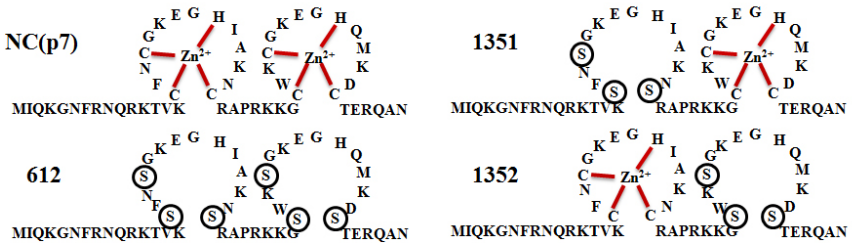

Fig. 1. The sequence of the Psi and RNA aptamers and the structure of the NC and NC mutants. (A) Wild type Psi and RNA aptamers sequence. The wild type Psi sequence was derived from the HIV ARV-2/SF2 strain and the RNA aptamer sequences are shown. (B) Amino acid sequence of the wild type and its zinc finger mutant NC protein derived from the HIV NL4-3 strain.

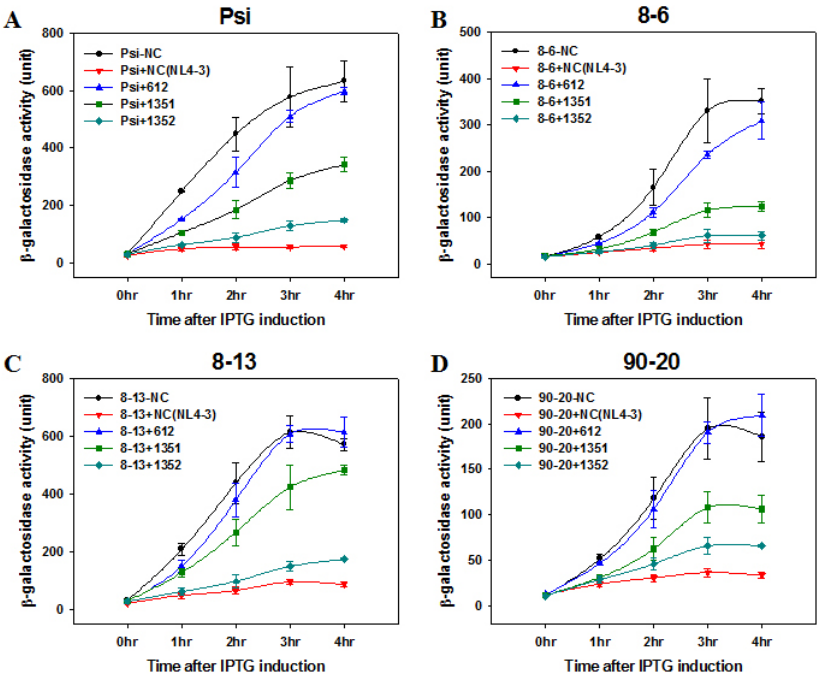

Fig. 2. β-galactosidase assay for the interaction of the RNA aptamers to the wild type and mutant NC protein. The *in vivo* binding affinity of each of the SELEX RNAs to NC was measured as described in Materials and Methods. In the case of the Ψ-RNA sequence (A), the reporter gene activity was repressed approximately 90-92% with the wild type NC, 15-20% with 1351, 70-72% with 1352, and 0-5% with 612. In the case of the 8-6 RNA sequence (B), the reporter activity was repressed approximately 85-90% with wild type NC, 64-66% with 1351, 83-84% with 1352, and 12-18% with 612. In the case of the 8-13 RNA sequence (C), the reporter activity was repressed approximately 84-86% with the wild type NC, 15-20% with 1351, 70-75% with 1352 and almost no repression with 612. In the case of the 90-20 RNA sequence (D), the reporter activity was repressed approximately 85-88% with the wild type NC, 50-55% with 1351, 69-72% with 1352 and almost no repression with 612. All experiments were performed in triplicate.

cells with and without the wild type NC as well as the NC zinc-finger mutant protein expression plasmids (pJC1/612, pJC1/1351, pJC1/1352), respectively. The wild type NC and its zinc-finger mutant NC protein were derived from a HIV NL4-3 isolate instead of an ARV-2 isolate that was used previously (22). The β-galactosidase translation inhibition activities resulting from each interaction between NC and the RNA aptamers as well as the NC mutant proteins and RNA aptamers were then measured as described in Materials and Methods.

In the case of Ψ-RNA as a control, the greatest inhibition of the reporter gene activity that represents the binding affinity between NC and Ψ was observed with the wild type NC. Each of NC zinc finger mutants shows varying degrees of binding affinity. The degree of inhibition of the β-galactosidase activity is NC > 1352 > 1351 > 612. This demonstrates that the zinc finger motif of the NC protein is important for recognizing Ψ-RNA. In particular, the first zinc finger domain is more important for specific binding to the Ψ-RNA sequences than the second zinc finger (Fig. 2A). This pattern of reporter gene activity is indicative of the specific interaction between NC and Ψ-RNA, as previously reported (22).

In the case of each RNA aptamer, the pattern of binding to the NC protein and NC zinc finger mutants is the same that with the Ψ-RNA (Fig. 2B, 2C, 2D). This means that all the aptamer RNAs examined here show the highest binding affinity with the wild type NC and the same dependency of the zinc finger motif of NC protein. The result of the β-galactosidase activity assay correlates well with the western blot data (Fig. 3). In order to further validate the assay system, another RNA aptamer was tested, namely SW8.4 RNA isolated previously by Clever *et al* (2000) (23). Again, the pattern of reporter gene activity with NC and the NC mutants is the same as that with Ψ-RNA and the other RNA aptamers (Fig. 4). This confirms that the cell-based assay developed for the NC-Ψ interaction can be further extended to NC-binding RNAs other than Ψ to measure its specific binding to NC.

It should be noted that not all the aptamers tested in this study had a higher binding affinity than Ψ, which is in contrast to that reported previously *in vitro* (Fig. 3A), where they were shown to have higher binding (at least 10-fold or higher) than Ψ RNA. Further studies will be needed to determined if this is because the structures of the aptamer RNAs *in vitro* do not properly reflect the *in vivo* condition, which are different within cells, or because of the difference in the assays used even though the translational repression activity in this cell-based assay was proportional to the binding affinity between the protein of interest and its interacting RNAs, as previously reported (22, 24). In this regard, these results suggest that it is necessary to further confirm the binding activity of the NC aptamer RNAs identified *in vitro* under living cell conditions or in an appropriate cell-based assay shown here, particularly if the RNA aptamers is intended to be used as an anti-viral agent against HIV in the future.

In summary, these results confirm for the first time the *in vivo* specific binding activity of SELEX NC aptamer RNAs identified *in vitro* and verify the utility and expandability of this cell-based assay to the interaction of NC and the NC specific RNAs.

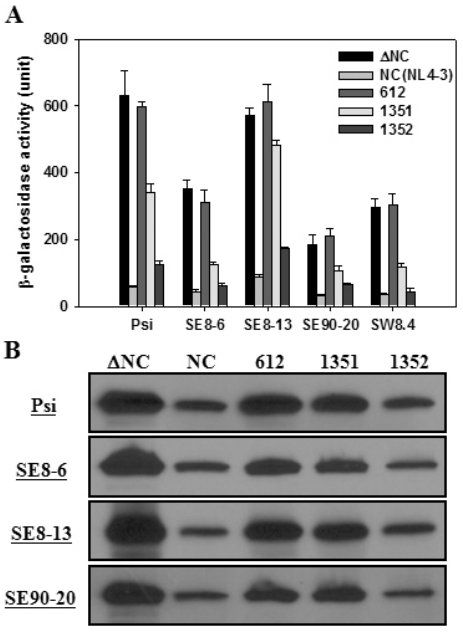

Fig. 3. Western blot and ONPG assay for Psi-NC, mutant NC interaction in *E.coli*. The levels of inhibition of β-galactosidase protein expression resulting from each RNA-NC interaction were determined by western blot analysis using 12% SDS-polyacryamide gel, as described in Materials and Methods. Shown are each of the samples obtained at 3hr after IPTG induction. (A) β-galactosidase activity of each RNA-NC interaction at 3 hrs after IPTG induction and (B) western blot results.

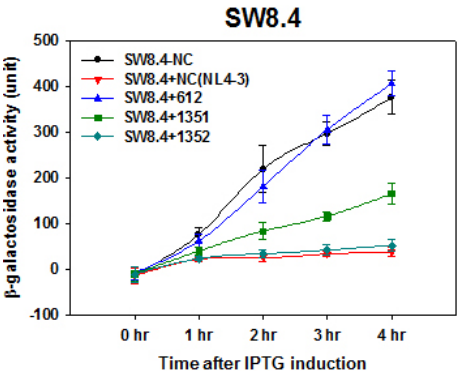

Fig. 4. β-galactosidase assay for the interaction of SW8.4 RNA aptamer to the wild type and mutant NC protein. Shown is the binding activity of SW8.4 to the wild type and mutant NC proteins determined, as described in Materials and Methods. The reporter gene activity was repressed approximately 88% with the wild type NC, 60% with 1351, 85% with 1352, and 0% with 612, respectively. The experiment was performed in triplicate.

MATERIALS AND METHODS

Plasmids construction

The plasmids containing the SELEX RNA aptamers, pMV/AS-SE8-6/LacZ, pMV/AS-SE8-13/LacZ and pMV/AS-SE90-20/LacZ, were constructed as follows. Each SELEX RNA fragment was generated by PCR amplification using pUC19/SE8-6, pUC19/SE8-13, and pUC19/SE90-20 (21). The SE8-6 sequence was amplified using the following primers: 5'-CAATACGTACGCG GAAGTACGACTGGGTA-3' and 5'-ATTCCTAGGACGGATAT CGGGAAGCGAGG-3'. The SE8-13 sequence was amplified by PCR using the following primers: 5'-CCCTACGTATATAGT GTGTTCAACGCTTA-3' and 5'-TGTCCTAGGCTCGTACAACG GACAAAAGT-3'. The SE90-20 sequence was amplified by PCR using the following primers: 5'-CCCTACGTAAATTGCAG TACGTAAAGTAT-3' and 5'-ATTCCTAGGCCATAGCGCATGT CCCAATG-3'. The PCR products of each SELEX aptamers were digested with *SnaB* I (Koschem, Korea) on the 5' terminal and *Avr* II (New England Biolabs, USA) on the 3' terminal (underlined), and then cloned into pMV/AS-Psi(Ψ)/LacZ, which had previously been optimized as a Ψ-independent plasmid (22), in place of the Ψ region by treating them with the same restriction enzymes. pMV/AS-SW8.4/LacZ was also constructed in a similar manner. The SW8.4 sequence shown in Fig. 1 was synthesized chemically by Genotech Inc. to contain the *SnaB* I site on the 5' terminal and the *Avr* II site on the 3' terminal (underlined). The SW8.4 oligomer was then inserted into pMV/AS13-Psi(Ψ)/LacZ using the *SnaB* I and *Avr* II restriction enzyme.

The NC and NC mutant plasmids were constructed as follows. The NC encoding sequence from the HIV NL4-3 isolate was amplified using the template, pLP1-NC (lab stock), and the following primers: 5'-CCAGATCTAGGAGGTTTAAAATAATG ATACAGAAAGGC-3' and 5'-ATTCTGCAGTCAATTAGCCTGT CTCTC-3'. The PCR product of NC was digested with *Bgl* II (Koschem, Korea) on the 5' terminal and *Pst* I (Roche, Switzerland) on the 3' terminal (underlined) and inserted into the pJC1 plasmid (25) using the same restriction enzyme. The NC mutant plasmids, pJC1/612, pJC1/1351 and pJC1/1352, were constructed using the same scheme for the NC plasmid shown above. Each NC zinc finger mutant, which has a replacement of cystein with serine, such as SSHS/SSHS (612), SSHS/CCHC (1351), CCHC/SSHS (1352) in place of the two original CCHC/CCHC zinc finger of wild type NC, were generated by PCR amplification using the template pDB612, pDB1351, pDB1352 (a gift of Dr. Gorelick in NCI, USA) and the same primers described above and then inserted into pJC1 using *Bgl* II and *Pst* I. All the constructs were confirmed by DNA sequencing.

Transformation

Each SELEX aptamer plasmid, pMV/AS-SE8-6/LacZ, pMV/AS-SE8-13/LacZ, pMV/AS-SE90-20/LacZ and pMV/AS-SW8.4/LacZ, was co-transformed with the pJC1(△NC), pJC1, pJC1/612, pJC1/1351 and pJC1/1352 in *E.coli*, JM109 strain (Promega Co., USA). In addition, the Psi-reporter vector, pMV/AS-Psi/LacZ, was transformed with each NC and NC mutant plasmid as a comparison. Subsequently, the double transformants were selected from the LB plate containing 100 μg/ml of ampicillin (USB, USA) and 10 μg/ml of tetracycline (Sigma Aldrich Co., USA)

β-galactosidase assay

The binding affinity of the SELEX aptamers for the NC protein was determined by measuring the β-galactosidase activity as described before (22). Briefly, the β-galactosidase activity was measured using the ONPG assay as follows. The double transformants were inoculated in liquid LB media containing ampicillin (100 μg/ml) and tetracycline (10 μg/ml) overnight at 37°C in a shaking incubator (250 rpm). On the next day, the transformants were sub-cultured in a 1/10 dilution in fresh liquid LB media containing the same antibiotics and then further incubated at 37°C in a shaking incubator until the OD600 reached 0.3 to 0.4. LacZ gene expression was induced by adding 1 mM isopropyl- β-D-thiogalactopyranoside (IPTG, Sigma) and incubated for 4 hours. The β-galactosidase activity was measured every hour. At the same time, the level of cell growth was determined by measuring the absorbance at 600 nm. 50 μl of each sample was harvested and lysed with 20 μl of 0.1% of SDS, 20 μl of chloroform and 450 μl of Z-buffer (0.06 M Na_2HPO_4, 0.04 M $NaHPO_4$, 0.01 M KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) and vortexed. The samples were incubated at RT for 5 min and 100 μl of *o*-nitrophenyl-D-thiogalactopyranoside (ONPG, Sigma Aldrich Co., USA) were added and left to stand at RT for 5 min. The reaction was quenched with 200 μl of 1 M Na_2CO_3 and centrifuged at 4°C, 13000 rpm for 5 min. 200 μl of the supernatant was used to measure the absorbance at 420 nm and 550 nm with a spectrophotometer (Infinite 200, TECAN, Austria).

Immunoblotting

At 3 hours after IPTG induction, samples containing an equal number of cells (OD600 = 1) were taken and centrifuged 12000 rpm for 1 min. The pellet was resuspended in 80 μl of sterile water and 20 μl 5X sample buffer (60 mM Tris-HCl, 2% SDS, 25% Glycerol, 0.1% bromophenol blue, β-mercaptoethanol) was then added. The samples were boiled for 10 min, centrifuged at 4°C at 14000 rpm for 20 min, and the supernatant was transferred to a new 1.5 ml tube. A 1/10 volume of the total protein was loaded in a 12% acrylamide gel and separated and transferred electrophoretically to a nitrocellulose filter membrane. A tris-Glycine electrophoresis buffer containing 20% methanol was added and an electric current of 350 mA was applied for 90 min. The membrane was blocked with 5% non-fat milk (BD, USA) in 1X Tris-buffered saline containing 0.05% tween20 for 40 min. The primary antibody was the anti β-galactosidase monoclonal antibody (Oncogene, USA) and the secondary antibody was HRP-Goat anti-mouse IgG conjugate (Zymed, USA).

Acknowledgments
This study was supported by a National Research Laboratory grant (M10500000148-06J0000-14810) from the Korean Ministry of Science and Technology and a research fund (R01-2005-000-11258) from Korea Science and Engineering Foundation.

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