Rapid Communication

J. Biochem. **134**, 637–639 (2003)
DOI: 10.1093/jb/mvg200

Role of the Zinc Fingers of HIV-1 Nucleocapsid Protein in Maturation of Genomic RNA

Seiki Baba[1], Ken-ichi Takahashi[*,1], Yoshio Koyanagi[2], Naoki Yamamoto[3], Hiroshi Takaku[1], Robert J. Gorelick[4] and Gota Kawai[†,1]

[1]Department of Industrial Chemistry, Faculty of Engineering, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino-shi, Chiba 275-0016; [2]Department of Virology, Tohoku University School of Medicine, 2-1 Seiryou-machi, Aoba-ku, Sendai 980-8575; [3]Department of Molecular Virology, Faculty of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519; and [4]dAIDS Vaccine Program, SAIC Frederick, Inc., NCI at Frederick, Frederick, Maryland 21702, USA

Received September 1, 2003; accepted September 25, 2003

The nucleocapsid protein of HIV-1 consists of two basic amino acid regions and two zinc fingers. We investigated the requirement of these domains for the structural conversion of a 39mer RNA covering the dimerization initiation site by using three peptides; wild-type NCp7, a mutant in which the two zinc fingers are mutated, and another mutant in which the two zinc fingers are deleted. The two mutants exhibited similar conversion activities to each other, which were lower than that of the wild-type, indicating that the two basic regions exhibit some activity for RNA chaperone, as we suggested before, and the zinc fingers enhance the efficiency of this activity.

Key words: DIS, HIV-1, maturation, nucleocapsid protein, zinc finger.

Abbreviations: HIV-1, human immunodeficiency virus type 1; DIS, dimerization initiation site; NC, nucleocapsid protein.

One of the functions of the nucleocapsid protein (NC) of human immunodeficiency virus type-1 (HIV-1) is as an RNA chaperone, which assists RNA molecules into their thermodynamically most stable conformation (*1*). NC consists of 55 amino acids (pNL4-3 sequence), of which basic residues, Arg and Lys, account for 27%. Another role of NC is in the recognition of genomic RNA during the assembly process (*2–5*). NC consists of two basic regions and two zinc fingers, as shown in Fig. 1A. A number of experiments have been performed to understand the role of the zinc fingers as well as the basic regions, and some of the results are summarized in Laughrea *et al*. 2001 (*6*). For example, mutations in the basic regions as well as the zinc fingers were found to inhibit the annealing of $tRNA^{Lys}_3$ to the primer binding site, whereas most site directed mutations, except for the distal (C-terminal) zinc finger, were silent as to genomic RNA dimerization. In contrast, the proximal (N-terminal) zinc finger was critically required for genomic RNA packaging. Recently, the CCHC fingers were found to be required for efficient nucleic acid chaperone activity including minus- and plus-strand transfer processes, as determined using a mutant NC having SSHS sequences instead of the native CCHC fingers (Fig. 1B) (*7*), and by using CCHH or CCCC NC mutants (*8*). On the other hand, the two basic regions of NC (Fig. 1C) can act as an RNA chaperone by converting the dimeric form of a 39mer RNA corresponding to the dimerization initiation site (Fig. 2) (*9*). It is difficult to compare these apparently contradictory results because the experiments were performed with different systems and conditions. Thus, it is important to compare the function with same system and conditions to examine the role of zinc fingers in the RNA chaperone activity of NC.

To examine the RNA chaperone activity of NC, we used the experimental system of a 39mer RNA (DIS39) covering the whole DIS sequence (*10*). DIS39 forms two types of dimers, the kissing-loop and extended-duplex dimers (Fig. 2), whose base pairing topologies have been confirmed by NMR analysis (*11*). They can be readily discriminated on PAGE: both dimers retain their dimeric states on electrophoresis through polyacrylamide gels containing Mg^{2+}. However, on PAGE without Mg^{2+}, the kissing-loop dimer separates into monomers, whereas the extended-duplex dimer remains in the dimeric state, because of their different Mg^{2+}-dependent stabilities. As reported in the previous paper, DIS39 mostly forms the kissing-loop dimer by itself at 37°C (Fig. 3, lanes 1 and 12) and is converted into the extended-duplex dimer when incubated at 55°C (lane 11).

We prepared three peptides, NCp7wt, NCp7-SSHS/SSHS and NCBR[1+2], which correspond to NCp7 of the pNL4-3 sequence, a mutant NCp7 having two SSHS sequences instead of the two CCHC zinc fingers, and the two basic regions of NCp7 of the LAV strain linked with two glycine residues (Fig. 1). DIS39 was converted into the extended-duplex dimer when incubated with equivalent or more NCp7wt at 37°C (Fig. 3, lanes 2–4). On the other hand, NCp7-SSHS/SSHS and NCBR[1+2] showed chaperone activities that were nearly identical and lower than that of NCp7wt (Fig. 3, lanes 5–7 and 8–10). This result indicates that the two basic regions surrounding

[*]Present address: Department of Bioscience, Faculty of Bioscience, Nagahama Institute of Bio-Science and Technology, 1266 Tamura-cho, Nagahama, Shiga 526-0829.
[†]To whom correspondence should be addressed. Tel/Fax: +81-47-478-0425, E-mail: gkawai@ic.it-chiba.ac.jp

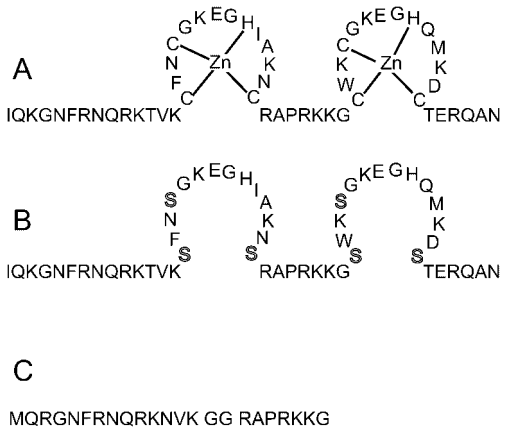

Fig. 1. **Peptide sequences of NCp7wt, NCp7-SSHS/SSHS and NCBR[1+2].** NCp7wt corresponds to NCp7 of the pNL4-3 sequence (A). NCp7-SSHS/SSHS has two SSHS sequences instead of the two CCHC zinc fingers of NCp7wt (B). NCBR[1+2] comprises the two basic regions of NCp7 of the LAV strain linked with two glycine residues (C). Recombinant NCp7wt and NCp7-SSHS/SSHS were expressed and purified as described previously (*7*, *17*, *18*). Synthetic peptide NCBR[1+2] was purchased from Sawady Technology (Tokyo).

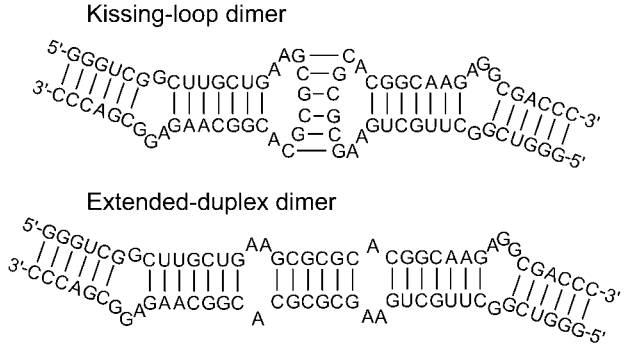

Fig. 2. **RNA sequence of a 39mer RNA corresponding to the dimerization initiation site (DIS39).** DIS39 forms two types of dimers, the kissing-loop and extended-duplex dimers, and NCp7 assists this conversion. RNA samples were synthesized enzymatically by *in vitro* transcription with AmpliScribe T7 Transcription Kits (Epicentre Technologies, WI, USA). Purification was performed by PAGE using 30 cm × 40 cm glass plates (Nihon Eido, Tokyo) under denaturing conditions, and extensive desalting by ultrafiltration (Centricon YM3., Amicon, MA, USA) was carried out.

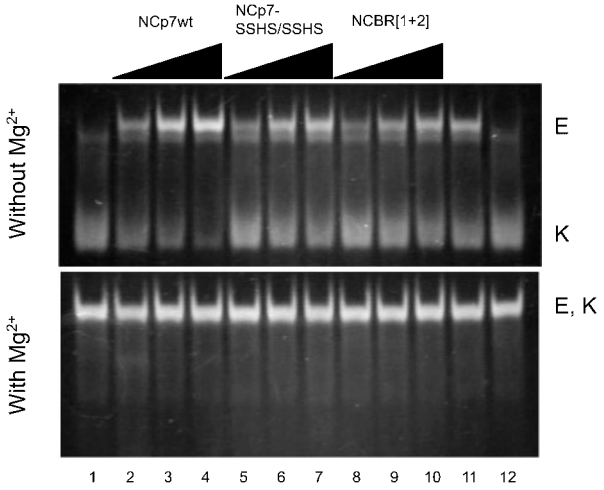

Fig. 3. **Assay for conformational conversion of DIS39 by NCp7wt, NCp7-SSHS/SSHS and NCBR[1+2].** Samples were incubated for 2.5 h at 37°C (lanes 1 to 10 and 12) or 55°C (lane 11). DIS39 was incubated alone (lanes 1, 11 and 12), or with 1, 2 and 3 equivalents of NCp7wt (lanes 2, 3 and 4, respectively), NCp7-SSHS/SSHS (lanes 5, 6 and 7, respectively) and NCBR[1+2] (lanes 8, 9 and 10, respectively). In the absence of Mg^{2+}, the kissing-loop dimer (K) separates into monomers, whereas the extended-duplex dimer (E) retains the dimeric state during electrophoresis through polyacrylamide gels. Both dimers retain their dimeric states in the presence of Mg^{2+}. The assay was performed as follows. After heating 12 μM DIS39 in 4 μl of water at 95°C for 5 min, it was chilled on ice for 5 min. Then 4 μl of 2 × PN buffer (1 × PN buffer contains 10 mM sodium phosphate [pH 7.0] and 50 mM NaCl) was added. After various concentrations of NCp7wt or its related peptides in 12 μl of 1 × PN buffer had been added, the RNA solutions were incubated at 37°C for 2.5 h and then treated with a phenol/chloroform solution regardless of the presence or absence of a peptide. Ten μl of the aqueous layer containing RNA was collected and mixed with 10 μl of loading buffer containing glycerol, and bromphenol and xylene cyanol dyes. The solution was then divided in two, which were separately analyzed by electrophoreses through nondenaturing polyacrylamide gels (10%) in TBM buffer (89 mM Tris, 89 mM borate, 1 mM $MgCl_2$) and TBE buffer (89 mM Tris, 89 mM borate, 2 mM EDTA), respectively, at room temperature. After electrophoresis the gels were stained with RedStain (BioRad Laboratories, CA, USA) and the RNA was visualized with an ultraviolet illuminator (FASIII., Toyobo, Osaka).

the N-terminal zinc finger has the RNA chaperone activity of NCp7 by themselves.

Although the NCp7wt sample used in the present study contained Zn^{2+} to prevent the formation of extended disulfide bridges within and among the molecules, we demonstrated that Zn^{2+} is not required for the efficient annealing activity in the DIS39 system in the previous study (*10*). Judging from the above together with the finding that NCp7-SSHS/SSHS and NCBR[1+2] still have annealing activity, the two zinc fingers might be important for defining the structural arrangement of the two basic regions in the case of the DIS39 system. Probably, the two basic regions in NCp7-SSHS/SSHS and NCBR[1+2] cannot form the optimized arrangement of the basic regions and/or residues for the chaperone activity. Some mutations in the basic regions as well as the zinc fingers, but not the zinc coordinating residues decrease the RNA chaperone activity for $tRNA^{Lys}_3$ annealing (*6*, *12*). This may also be due to the disruption of the optimized arrangement of the basic regions and/or residues. Probably because the process of conversion of genomic RNA dimeric forms is simpler than that of $tRNA^{Lys}_3$ annealing, the effects of mutations on the dimerization are less drastic than these on the $tRNA^{Lys}_3$ annealing.

The NC domain of the Gag polyprotein has been found to tightly bind to the genomic RNA dimer via SL2 and SL3 stem-loops just downstream of the DIS (SL1) region (*3*, *13*). Thus, during virus particle formation, the zinc fingers are involved in recognizing the genomic RNAs in the first step and then, after cleavage of the Gag polyprotein (*14*, *15*), the basic regions of NCp7 induce the conformational change of the genomic RNA dimer, which is optimized by the presence of the zinc finger regions. The

present study showed that the DIS annealing activity, which reflects the conversion efficiency of the conformation of DIS, of NCp7 was also decreased to roughly 10% by the SSHS/SSHS mutation, judging from the band densities on the gel (Fig. 3). On the other hand, a previous study showed that the SSHS/SSHS mutation resulted in a <10% wild-type RNA content in the virus (*7*), suggesting a possible relation between the packaging of genomic RNAs and DIS annealing. It should be noted that deletion of the DIS (SL1) region also resulted in a 10% wild-type RNA content in the virus and the mutant virus exhibited 10% infectivity (unpublished results). Darlix *et al*. also suggested that RNA dimerization and packaging are related events (*5*, *13*), whereas Sakuragi *et al*. showed that dimerization can be dissociated from packaging (*16*). This possibility should be verified through future research.

GK wishes to thank Dr. Judith G. Levin for providing the authors the chance of this collaboration. This work was supported by the "Research for the Future" Program (JSPS-RFTF97L00503) of the Japan Society for the Promotion of Science, and by, in part, a Grant-in-Aid for High Technology Research from the Ministry of Education, Science, Sports and Culture, Japan, and, in part, federal funds from the National Cancer Institute, National Institutes of Health, under contract No. NO1-CO-12400 with SAIC-Frederick, Inc.

REFERENCES

1. Rein, A., Henderson, L.E., and Levin, J.G. (1998) Nucleic-acid-chaperone activity of retroviral nucleocapsid proteins: significance for viral replication. *Trends Biochem. Sci.* **23**, 297–301
2. de Guzman, R.N., Wu, Z.R., Stalling, C.C., Pappalardo, L., Borer, P.N., and Summers, M.F. (1998) Structure of the HIV-1 nucleocapsid protein bound to the SL3 Ψ-RNA recognition Element. *Science* **279**, 384–388
3. Amarasinghe, G.K., de Guzman, R.N., Turner, B.G., Chancellor, K.J., Wu, Z.R., and Summers, M.F. (2000) NMR structure of the HIV-1 nucleocapsid protein bound to Stem-Loop SL2 of the Ψ-RNA packaging signal. Implications for Genome recognition. *J. Mol. Biol.* **301**, 491–511
4. Berkowitz, R., Fisher, J., and Goff, S.P. (1996) RNA packaging. *Curr. Top. Microbiol. Immunol.* **214**, 177–218
5. Darlix, J.L., Lopez-Lastra, M., Mély, Y., and Roques, B. (2003) Nucleocapsid protein chaperoning of nucleic acids at the heart of HIV structure, assembly and cDNA synthesis in *HIV Sequence Compendium 2002* (Kuiken, C., Foley, B., Freed, E., Hahn, B., Marx, P., McCutchan, F., Mellors, J.W., Wolinsky, S., and Korber, B., eds.) pp. 69–88, Los Alamos National Laboratory, Los Alamos, NM
6. Laughrea, M., Shen, N., Jette, L., Darlix, J., Kleiman, L., and Wainberg, M.A. (2001) Role of distal zinc finger of nucleocapsid protein in genomic RNA dimerization of human immunodeficiency virus type 1; No role for the palindrome crowning the R-U5 hairpin. *Virology* **281**, 109–116
7. Guo, J., Wu, T., Anderson, J., Kane, B.F., Johnson, D.G., Gorelick, R.J., Henderson, L.E., and Levin, J.G. (2000) Zinc finger structures in the human immunodeficiency virus type 1 nucleocapsid protein facilitate efficient minus- and plus-strand transfer. *J. Virol.* **74**, 8980–8988
8. Guo, J., Wu, T., Kane, B.F., Johnson, D.G., Henderson, L.E., Gorelick, R.J., and Levin, J.G. (2002) Subtle alterations of the native zinc finger structures have dramatic effects on the nucleic acid chaperone activity of human immunodeficiency virus type 1 nucleocapcid protein. *J. Virol.* **76**, 4370–4378
9. Takahashi, K., Baba, S., Koyanagi, Y., Yamamoto, N., Takaku, H., and Kawai, G. (2001) Two basic regions of NCp7 are sufficient for conformational conversion of HIV-1 dimerization initiation site from kissing-loop dimer to extended-duplex dimer. *J. Biol. Chem.* **276**, 31274–31278
10. Takahashi, K., Baba, S., Chattopadhyay, P., Koyanagi, Y., Yamamoto, N., Takaku, H., and Kawai, G. (2000a) Structural requirement for the two-step dimerization of human immunodeficiency virus type 1 genome. *RNA* **6**, 96–102
11. Takahashi, K., Baba, S., Hayashi, Y., Koyanagi, Y., Yamamoto, N., Takaku, H., and Kawai, G. (2000b) NMR analysis of intra- and inter-molecular stems in the dimerization initiation site of the HIV-1 genome. *J. Biochem.* **127**, 681–686
12. Remy, E., Rocquigny, H., Petitjean, P., Muriaux, D., Theilleux, V., Paoletti, J., and Roques, B.P. (1998) The annealing of $tRNA^{Lys}_3$ to human immunodeficiency virus type 1 primer binding site is critically dependent on the NCp7 zinc fingers structure. *J. Biol. Chem.* **273**, 4819–4822
13. Darlix, J.L., Gabus, C., Nugeyre, M.T., Clavel, F., and Barre-Sinoussi, F. (1990) *Cis* elements and *trans*-acting factors involved in the RNA dimerization of the human immunodeficiency virus HIV-1. *J. Mol. Biol.* **216**, 689–699
14. Fu, W. and Rein, A. (1993) Maturation of dimeric viral RNA of moloney murine leukemia virus. *J. Virol.* **67**, 5443–5449
15. Fu, W., Gorelick, R.J., and Rein, A. (1994) Characterization of human immunodeficiency virus type 1 dimeric RNA from wild-type and protease-defective virions. *J. Virol.* **68**, 5013–5018
16. Sakuragi, J., Iwamoto, A., and Shioda, T. (2002) Dissociation of genome dimerization from packaging functions and virion maturation of human immunodeficiency virus type 1. *J. Virol.* **76**, 959–967
17. Carteau, S., Gorelick, R.J., and Bushman, F.D. (1999) Coupled integration of human immunodeficiency virus type 1 cDNA ends by purified integrase *in vitro*: Stimulation by the viral nucleocapsid protein. *J. Virol.* **73**, 6670–6679
18. Williams, M.C., Rouzina, I., Wenner, J.R., Gorelick, R.J., Musier-Forsyth, K., and Bloomfield, V.A. (2001) Mechanism for nucleic acid chaperone activity of HIV-1 nucleocapsid protein revealed by single molecule stretching. *Proc. Natl Acad. Sci. USA* **98**, 6121–6126