

Investigation of the effect of SRSF9 overexpression on HIV-1 production

Ga-Na Kim, Kyung-Lee Yu, Hae-In Kim & Ji Chang You*

Department of Pathology, National Research Laboratory for Molecular Virology, College of Medicine, The Catholic University of Korea, Seoul 05505, Korea

Serine-arginine-rich splicing factors (SRSFs) are members of RNA processing proteins in the serine-arginine-rich (SR) family that could regulate the alternative splicing of the human immunodeficiency virus-1 (HIV-1). Whether SRSF9 has any effect on HIV-1 regulation requires elucidation. Here, we report for the first time the effects and mechanisms of SRSF9 on HIV-1 regulation. The overexpression of SRSF9 inhibits viral production and infectivity in both HEK293T and MT-4 cells. Deletion analysis of SRSF9 determined that the RNA regulation motif domain of SRSF9 is important for anti-HIV-1 effects. Furthermore, overexpression of SRSF9 increases multiple spliced forms of viral mRNA, such as Vpr mRNA. These data suggest that SRSF9 overexpression inhibits HIV-1 production by inducing the imbalanced HIV-1 mRNA splicing that could be exploited further for a novel HIV-1 therapeutic molecule. [BMB Reports 2022; 55(12): 639-644]

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus family that has two copies of single-stranded, positive-sense RNA. During infection, HIV-1 produces its DNA product from the genomic RNA using a reverse transcriptase which is integrated into the infected host cell DNA. Then, the proviral DNA is transcribed into unspliced, 9 kb-sized, full-length mRNAs and over 40 different spliced pre-mRNAs produced by the alternative splicing process (1, 2). These products are largely classified into three categories according to their size: (i) unspliced mRNA (~9 kb), (ii) single-spliced mRNA (~4 kb), and (iii) multiple-spliced mRNA (~2 kb). In addition, ~2 kb mRNAs are abundant during early infection, while full-length mRNAs

(9 kb) and ~4 kb appear at the late stage of infection.

Viral proteins, such as Tat and Rev, which are produced from the translation of fully spliced mRNAs, can activate the transcription enhancement and cytoplasmic migration of partially and/or unspliced mRNAs from which other viral proteins like Gag or Pol are derived, and virus production is achieved by them (3, 4). These indicate that HIV-1 requires highly sophisticated mRNA processing by alternative splicing regulatory mechanisms for an efficient viral replication and production.

The alternative splicing of HIV-1 mRNA is regulated by several splicing factors in host cells. The members of the family of serine-arginine-rich splicing factors (SRSFs) are one of the important pre-mRNA alternative splicing factors in mammalian cells as a key regulator of gene expression (5). In general, SRSF proteins consist of an RNA regulation motif (RRM) domain interacting with RNA or other proteins, and an arginine-serine domain, which is rich in arginine and serine residues. They contribute to several steps of mRNA processing, including metabolism, splicing, export, and recruitment of splicing machines (6).

Previous studies on SRSF proteins for HIV-1 gene expression regulation showed that they are involved in steps of splicing. The HIV-1 mRNA has four 5'-splice donor sites (D1-4) and nine 3'-splice acceptor sites (A1, A2, A4c, A4b, A5, A6, A7); near these sites include exon splice enhancers (ESEs) and exon splice silencers (ESSs) that could efficiently control splicing. ESS sequences interact with heterogeneous ribonucleoprotein particles (hnRNPs), while ESE sequences bind to SRSF proteins that activate splicing and recruitment spliceosome (7-9). Thus, the interaction between SRSF proteins and ESEs according to the location of the splicing site regulates the expression of nine major viral proteins that are important to produce HIV-1 regulatory and accessory proteins.

The SR protein families SRSF2, SRSF5, SRSF6, and SRSF7 compete with hnRNP and bind to ESE near A3 (10, 11). It was reported that these could inhibit HIV-1 production by regulating Tat expression. Moreover, SRSF4 promotes splicing and regulates Vif expression by binding ESE downstream of A1 (12). The interaction of SRSF1 and SRSF5 with the downstream ESE of A5, which promotes snRNP binding to D4, regulates Rev, Vpu, Env, and Nef expression (13, 14). Furthermore, SRSF1 inhibits HIV-1 production by inducing aberrant splicing by

*Corresponding author. Tel: +82-2-3147-8734; Fax: +82-2-3147-9282; E-mail: jiyou@catholic.ac.kr

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binding to ESEs near the A2 splices accept site, leading to an increase in Vpr mRNA (15).

SRSF9 (alternatively called SRp30c), one of the SR protein family, is also involved in the pre-mRNA splicing mechanism in mammalian cells. It was reported that it could control L1 mRNA expression by splicing directly and indirectly in human papillomavirus type 16 (16, 17). However, the effect of SRSF9 on HIV-1 production and splicing of gene regulation has not been reported thus far.

In this study, we have identified possibly for the first time that the SRSF9 overexpression inhibits HIV-1 production via inducing the aberrant alternative splicing of HIV-1 mRNA. Therefore, SRSF9 is indeed another novel cellular factor affect to HIV-1 mRNA splicing; it could be exploited as a molecule for the development of anti-HIV-1 therapy.

RESULTS

SRSF9 overexpression inhibits HIV-1 production

To investigate whether SRSF9 affects HIV-1 production, the Flag-tagged SRSF9 expression vector was co-transfected with proviral plasmid pNL4-3_{GFP} to HEK293T cells. After 24 h, the viral production levels in cell lysates were confirmed by Western blotting using the p24 antibody, which detects the capsid protein of HIV-1 as an expression marker of the virus. The levels of virus particle production in supernatants were measured by p24 ELISA assay. It was found that the production of viral particles decreased significantly as the SRSF9 concentration increased compared to that in the control (Flag-tagged empty vector) (Fig. 1A). The expression and processing levels of p55^{Gag} protein in the cells were also decreased by SRSF9 overexpression (Fig. 1B).

To further confirm the inhibitory effects of SRSF9 on viral production, MT-4 cells, a human CD4⁺ T cell line, were infected

with the same volume of each viral supernatant obtained from the transfection assay performed as above. After 72 h, the GFP expression levels were measured by fluorescence microscopy and quantified by FACS analysis. The results showed that GFP expression levels, which is an indicator of the number of infectious viruses in a supernatant, decreased proportionally with the SRSF9 concentrations (Fig. 1C, D). These data showed that SRSF9 overexpression inhibits HIV-1 production dose-dependently.

Effect of overexpressed SRSF9 on HIV-1 production in MT-4 cells

To examine the effect of SRSF9 in the whole HIV-1 life cycle, we examined the effect of HIV-1 production according to SRSF9 expression in virus-susceptible MT-4 cells. The MT-4 cells were transfected with an SRSF9 expression vector and then infected with an equal multiplicity of infection (MOI) of pNL4-3_{GFP}-derived viruses. We examined the overexpression of SRSF9 in cells using Western blotting (Fig. 2A). To confirm the SRSF9 effect of viral replication on MT-4 cells, GFP expression levels were measured after 72 h via fluorescence and quantified using FACS analysis. The results showed that the overexpression of SRSF9 inhibits viral production (Fig. 2B, C). Overall, these data indicated that the production and replication of HIV-1 were negatively affected by the overexpression of the SRSF9.

SRSF9 depletion increases the production of HIV-1

To determine that effects of SRSF9 depletion on viral production, HEK293T cells were transfected with small interfering RNAs (si-RNAs) of the control and SRSF9. Then, the control (nontarget siRNA) and SRSF9 knock-down cells were transfected to pNL4-3_{GFP}. After 24 h, cell lysates were subjected to Western blotting, and supernatants were examined by Western

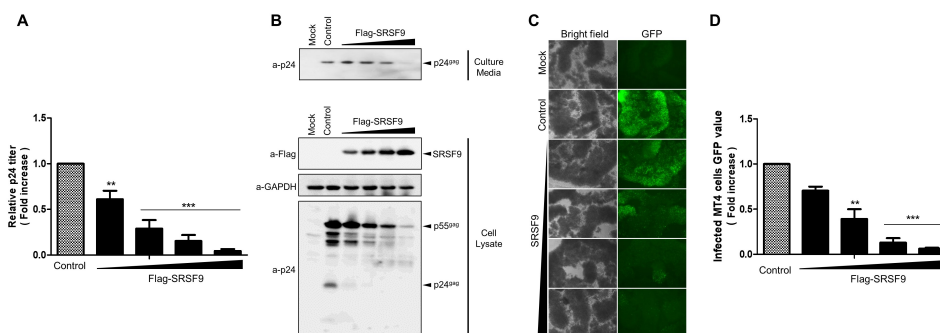


Fig. 1. SRSF9 overexpression inhibits HIV-1 production. HEK293T cells were co-transfected with 1 µg pNL4-3_{GFP} and 125 ng, 250 ng, 500 ng, or 1 µg flag-tagged SRSF9 expression vectors. As the control, a flag-tagged empty vector was used to equal the total amount of transfected DNA. After 24 h, virus production levels were measured by p24 enzyme-linked immunosorbent assay (p24 ELISA) in cell supernatants (A). The viral protein expression levels in cell lysates and supernatants were verified by Western blot (B). MT-4 cells were infected with the same volume of virus-containing culture supernatants. After 72 h, GFP levels were observed using a fluorescent microscope (C) and quantified by FACS analysis (D). The asterisks indicate a significant difference from control, analyzed by one-way ANOVA (*P < 0.05, **P < 0.005, and ***P < 0.001).

blotting and p24 ELISA assay. The results indicated that the production of viral particles in SRSF9 knock-down HEK293T cells was slightly increased compared to that in the control (Supplementary Fig. 1A). However, in corresponding cells, the same increased viral proteins were not observed as shown (Supplementary Fig. 1B).

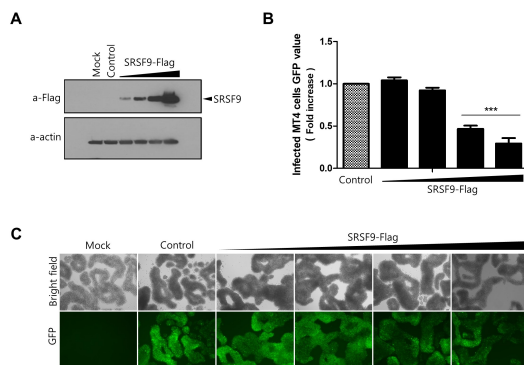


Fig. 2. Effect of overexpressed SRSF9 on HIV-1 production in MT-4 cells. MT-4 cells were transfected with 125 ng, 250 ng, 500 ng, or 1 μ g flag-tagged SRSF9 expression vector. After 24 h post-transfection, cells were infected with same MOI (200 pg) produced in HEK293T cells by pNL4-3_{GFP} for three days. The cell lysates were subjected to Western blot (A) and the GFP levels were quantified via FACS analysis (B) and measured by fluorescent microscope (C). The asterisks indicate a significant difference from control, analyzed by one-way ANOVA (* $P < 0.05$, ** $P < 0.005$, and *** $P < 0.001$).

To further confirm the effects of SRSF9 depletion, MT-4 cells were infected with each viral supernatant acquired in the transfection assay. The GFP expression levels were measured after 72 h by fluorescence. The GFP signals of MT-4 cells infected with the same volume of supernatants in the knock-down SRSF9 were co-related with Supplementary Fig. 1A (Supplementary Fig. 1C). These results demonstrated that SRSF9 depletion could enhance HIV-1 production.

The RRM domain of SRSF9 is required for HIV-1 inhibition

To determine which domain of SRSF9 was responsible for the observed antiviral effect, a number of expression vectors were generated for various truncates of SRSF9, as schematically shown in Fig. 3A. Each truncate was co-transfected with pNL4-3_{GFP} into HEK293T cells; then, the production of viruses was compared to the wild type (WT) of SRSF9 in supernatants by Western blotting and p24 ELISA. While Δ RS, which has a low rich arginine-serine domain showing an anti-HIV-1 effect is not different from that in WT SRSF9, both Δ RRM2-RS and Δ RRM1 showed either a negligible or no inhibition of virus production at the p24 levels of the supernatant (Fig. 3B). To determine the effect of each truncate on virus production, the supernatants were infected to MT-4 cells with the same volume; the viral infectivity was analyzed by GFP fluorescence. The GFP levels were reduced in WT and Δ RS but were not with either Δ RRM2-RS or Δ RRM1 (Fig. 3C). The data indicate that the RRM domain of SRSF9 is important and necessary for HIV-1 inhibition.

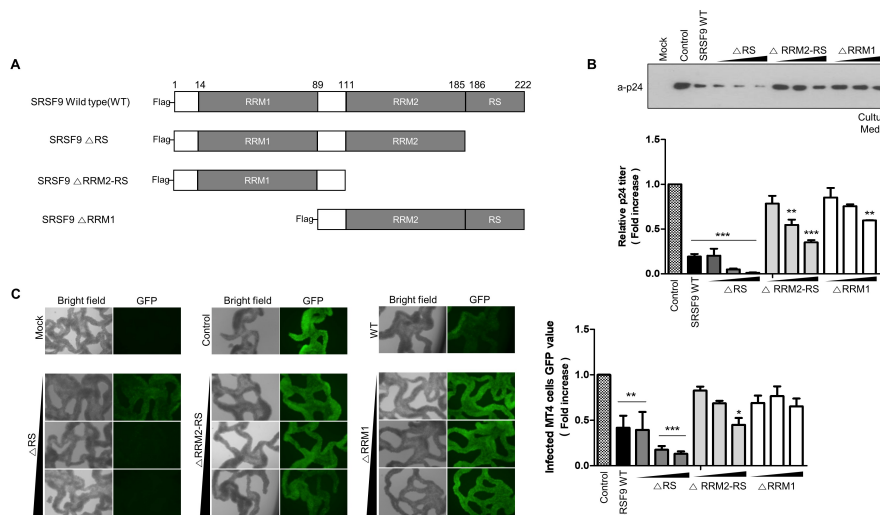


Fig. 3. The RRM domain of SRSF9 is required for HIV-1 inhibition. The schematic diagram of SRSF9 truncates (A). HEK293T cells were co-transfected with 1 μ g of pNL4-3_{GFP} with 250 ng of WT or 250 ng, 500 ng, or 1 μ g of each truncate of SRSF9. Then, 24 h post-transfection, the cell supernatants were evaluated by Western blot (upper) and p24 ELISA (lower) (B). MT-4 cells were transfected with the same volume of supernatants for three days, and the GFP levels were analyzed by fluorescent microscopy (left) and FACS analysis (right) (C). The asterisks indicate a significant difference from control, analyzed by one-way ANOVA (* $P < 0.05$, ** $P < 0.005$, and *** $P < 0.001$).

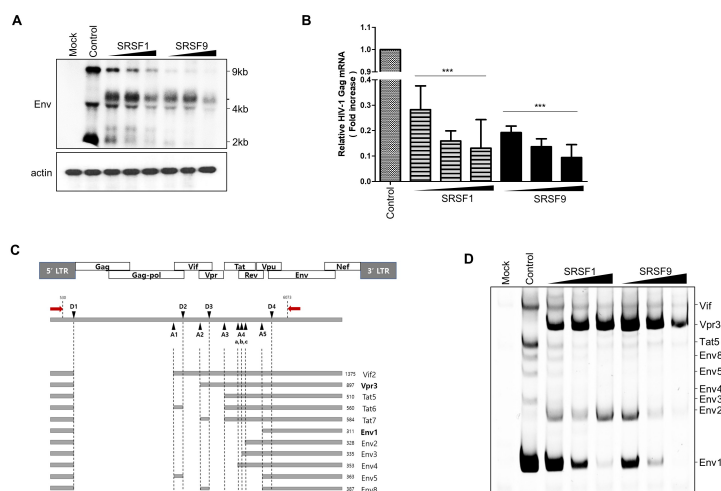


Fig. 4. The overexpression of SRSF9 alters the alternative splicing pattern of HIV-1 mRNA. HEK293T cells were co-transfected with 1 μ g of pNL4-3_{GFP} with Flag-tagged SRSF1 or Flag-tagged SRSF9. After 24 h, 1.5 μ g of the extracted RNA was subjected to Northern blot using a DIG-labeled Env probe (A) and RT-qPCR assay using a Gag primer (B). The extracted RNA was subjected to RT-PCR using primers (presented with red arrows) to detect -4 kb mRNAs, and the produced -4 kb mRNA RT-PCR products are presented in the scheme (C). PCR products were separated on 6% denaturing polyacrylamide gel (D). The asterisks indicate a significant difference from control, analyzed by one-way ANOVA (*P < 0.05, **P < 0.005, and ***P < 0.001).

The overexpression of SRSF9 alters the alternative splicing pattern of HIV-1 mRNA

A previous study showed that SRSF proteins in mammalian cells regulate the HIV-1 mRNA splicing process, which is significant for viral protein production and infectivity. Therefore, we examined how SRSF9 affects the splicing of viral mRNA in comparison to that of SRSF1 as an HIV-1 inhibitor by activating splicing that strongly increased Vpr mRNA (15). The pNL4-3_{GFP} and SRSF9 or SRSF1 were transfected into HEK293T cells, and the RNA was extracted after 24 h. The extracted RNA was subjected to RT-PCR using a Gag primer, and to Northern blot using a DIG-labeled Env probe, which can detect both unspliced mRNA (9 kb) and spliced mRNA (4 kb, 2 kb). SRSF9 overexpression resulted in abnormally spliced products (~5 kb mRNA) compared to the control group, as shown with SRSF1 (Fig. 4A), and the level of 9 kb mRNA was significantly decreased in the SRSF1 and SRSF9 groups. In addition, SRSF9 overexpression appeared to decrease dose-dependently the Gag mRNA level (Fig. 4B). These results suggest that SRSF9 overexpression induced an aberrant splicing process as SRSF1 overexpression.

To further identify the splicing of HIV-1 mRNA by SRSF9, the splicing products were examined by RT-PCR using primers to detect -4 kb mRNAs (the primers used are presented with red arrows in Fig. 4C). HEK293T cells were transfected with pNL4-3_{GFP} and the expression vectors of SRSF1 and SRSF9. After 24 h, the RNA was extracted, and RT-PCR products were confirmed. The overexpressed SRSF1 and SRSF9 showed significant differences in splicing products compared to those in the control. In the overexpressed SRSF9 group, the products of Vpr mRNA

were increased such as SRSF1 overexpressed, by activating the A2 splices accept site, compared to the controls (Fig. 4D) (10). In addition, Env mRNAs which are represented mostly as the 4-5 kb mRNAs were reduced. These results suggested that SRSF9 overexpression alters the splicing pattern of HIV-1, as observed in the case of SRSF1.

Taken together, SRSF9 is novel cellular factor enabling the effect to the alternative splicing of HIV-1 mRNA, and overexpression of SRSF9 could inhibit HIV-1 production.

DISCUSSION

Although many anti-HIV drugs were developed for acquired immunodeficiency syndrome, it is still a global health problem (18). Therefore, it is important to further understand the molecular mechanisms of virus replication and the possible targets and mechanisms of virus inhibition. In the HIV-1 life cycle, alternative splicing and intracellular splicing are important to generate various viral proteins (19).

SRSF proteins are involved in the splicing of HIV-1 mRNA and are expressed as SRSF1-7, 9, and 11 by nine genes; most of these have been studied on various HIV-1 subjects (8). The SRSF protein could affect several stages in HIV-1 life cycles, including alternative splicing, exporting to nuclear, transcription, and translation of Gag. The overexpression of SRSF1, SRSF2, SRSF3, and SRSF7 reportedly inhibits Tat-LTR mediated transcription (20, 21). In addition, they could activate the nuclear export of mRNA and translation (22-24). Reports have indicated that SRSF1-7 has negative effects on the production of infectious HIV-1 virion (11, 15, 25, 26). Despite many studies

of SRSF proteins on HIV-1, the effect of SRSF9, another SR protein family, on HIV-1 replication and production is still unknown. In the present study, for the first time, the effect of SRSF9 on HIV-1 production was examined. The overexpression of SRSF1, SRSF2, or SRSF5 is known to strongly inhibit viral production and infectivity in HEK293T cells, our findings show concededly that SRSF9 overexpression inhibits HIV-1 production (Fig. 1).

The effects of most SRSF proteins on HIV-1 have been analyzed using HeLa or HEK293T cells. However, HIV-1 infects human CD4⁺ T cells and causes AIDS, and the effect of overexpressed SRSF proteins in these cells requires further elucidation (27). Therefore, we examined the effects of overexpressed SRSF9 on the HIV-1 life cycle using human T cell lines (MT-4 cells). Our results clearly showed that SRSF9 negatively affects the production of HIV-1 in human T cells as well as HEK293T cells (Fig. 2).

Knock-down studies of SRSF proteins showed conflicting results on HIV-1 production. SRSF1 and SRSF2 promoted the production of HIV-1 virion and infectivity; however, SRSF5 inhibits HIV-1 production and infectivity in HEK293T cells (26). Furthermore, we have examined the effect of knock-down of SRSF 9 protein. Interestingly, our results showed that the depletion of SRSF9 proteins only increased viral production slightly (Supplementary Fig. 1). In general, there are many SRSF proteins in cells which have a redundant function (6, 28). Therefore, it is quite expected and reconciled that even if knock-down of SRSF9 protein, the function of other SRSF proteins could still be supplemented even in a condition of depletion of SRSF9 in cells.

SRSF proteins have a rich arginine-serine domain and at least one RRM domain (29). Furthermore, the SRSF9 proteins have three domains, including the RRM homolog (RRMH). The domain deletion analysis results have demonstrated that the Δ RRM truncate could not inhibit HIV-1 production (Fig. 3). The SRSF1 and SRSF9 proteins consist of one RS domain and two RRMH domains and are similar in length (30). Therefore, the RRM domain of SRSF9 showed the same function as that of SRSF1 which demonstrated a strong HIV-1 inhibitory effect in previous reports (31). Considering that the RRM domain is known as a direct N-terminal RNA binding motif, it is expected to be a major domain for the observed induced aberrant splicing of HIV-1 mRNA by SRSF9 (32).

The SRSF1, which was used as a control in this study, plays a role in mammalian cells to carefully splice and regulate splicing in the SR protein family. The SRSF1 proteins could affect various steps in HIV-1 replication, and alternative splicing studies account for the largest part of the HIV-1-related functions presented by these proteins. In alternative splicing of HIV-1 mRNA, the SRSF1 regulates Vpr mRNA expression by binding to ESE1 near the SA2 splicing accept sites. Therefore, overexpression of SRSF1 disrupt the balance of alternative splicing of viral mRNA, inhibits Gag and Env protein synthesis, and consequently, virion production (15). The sequences and structural similarities between SRSF1 and SRSF9 are high accord-

ing to the phylogenetic tree of the SR protein family aligned by mafft, v7, L-INS-I method (33). This, SRSF9 could also regulate alternative splicing.

Our results showed that SRSF9 overexpression produced abnormal splicing products near 4-5 kb mRNAs, very similar to the case of SRSF1 (Fig. 4), decreased the level of Gag mRNAs by reducing the 9 kb mRNA. In addition, SRSF9 showed the same splicing product pattern as SRSF1, which was known to alter the splicing pattern of HIV-1 mRNA (increases the Vpr mRNA and decreases Env mRNA) (10,15). Notably, with the increase in SRSF9 concentration, a decrease in total mRNA levels was observed. These data suggested that an overall amount of the target substrate mRNA was reduced through excessive splicing activity of SRSF9 as observed with the anti-viral effect of the previously reported SRSF protein (15). Therefore, the reduction of unspliced viral mRNA in SRSF9 overexpressed cells leads to a negative effect on the production of virions.

These findings showed clearly that SRSF9 overexpression inhibits HIV-1 production and overexpressed SRSF9 alters the splicing pattern that increases the Vpr splicing product of HIV-1 mRNAs, suggesting that SRSF9 is a new cellular splicing factor effect to the alternative splicing of HIV-1 mRNA.

MATERIALS AND METHODS

Detailed information on Materials and methods is available in the supplementary section.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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