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ScienceDirect
Antiviral Research 79 (2008) 136–141
Antiviral Research
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Short communication

Creation of a bi-directional protein transduction system for suppression of HIV-1 expression by p27SJ

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Received 30 August 2007; accepted 19 November 2007

Abstract

p27SJ is a novel protein from a callus culture of St. John's wort that modulates transcription of the HIV-1 promoter in several mammalian cells [Darbinian-Sarkissian, N., Darbinyan, A., Otte, J., Radhakrishnan, S., Sawaya, B.E., Arzumanyan, A., Chipitsyna, G., Popov, Y., Rappaport, J., Amini, S., Khalili, K., 2006. p27(SJ), a novel protein from St. John's wort, that suppresses expression of HIV-1 genome. Gene Ther. 13, 288–295]. Here, we armed p27SJ with signals from Ig-kappa light chain that allow its efficient excretion from the cells, and from HIV-1 Tat that facilitates its uptake by other cells for its utilization by a protein transduction method. We demonstrate that treatment of cells containing the HIV-1 LTR with conditioned media from cells expressing the armed p27SJ ($_{exc}$p27SJ$_{upt}$) results in suppression of the viral activation by the C/EBPβ transcription factor. Once imported into the cells, $_{exc}$p27SJ$_{upt}$ impacts the nuclear localization of C/EBPβ and by retaining the protein in the cytoplasm affects its DNA binding and hence transcriptional activity. The armed p27SJ also inhibits Tat-induced activation of the LTR and decreases the level of viral replication in promonocytic cells including U-937 and T-lymphocytic cells. Our observations introduce a new bi-directional protein transduction system with a broad spectrum of applications for manufacturing therapeutic peptides by a specific group of cells called donor, and delivery to the target cells named recipient. Furthermore, our results support the utility of soluble p27SJ in suppressing transcription and replication of HIV-1 by interfering with the function of cellular proteins such as C/EBPβ and viral activators including Tat.

Keywords: HIV-1; St. John's wort

Previously we purified a peptide from total protein extract from a callus culture of *Hypericum perforatum* and based on its partial sequence data, we isolated a cDNA fragment encoding a 27 kDa protein named p27SJ (Darbinian-Sarkissian et al., 2006). Complete amino acid sequencing of the protein showed the presence of a characteristic DINGG motif at the N-terminal region suggesting that p27SJ may belong to an emerging family of DING proteins (Berna et al., 2007). In humans, a peptide containing DING was first determined in synovial fluid that was believed to be part of a larger protein of p205 synovial T-cell stimulating protein (Blass et al., 1999; Hain et al., 1996). Subsequent studies led to the identification of another member of the human DING family with growth-promoting effects in normal and tumor cells (Adams et al., 2002; Belenky et al., 2003; Morales et al., 2006). In addition to human tissue, DING proteins have been identified in various animal and plant tissues, and exhibit close homology with pseudomona proteins (for review see Ahn et al., 2007; Berna et al., 2002, 2007; Chen et al., 2007; Lewis and Crowther, 2005; Moniot et al., 2007; Pantazaki et al., 2007; Riah et al., 2000; Scott and Wu, 2005). Similar to its bacterial counterpart, p27SJ has the phosphate-binding residue, although its ability to interact with phosphate and its biological significance have remained unclear. Ectopic expression of p27SJ in various eukaryotic cells led to accumulation of this protein in the perinuclear cytoplasmic compartment. Moreover, p27SJ exhibits the ability to interact with several cellular proteins including C/EBPβ and viral proteins such as HIV-1 Tat (Darbinian-Sarkissian et al., 2006). As both proteins play a critical role in the activation of the HIV-1 genome, we investigated the impact of p27SJ on transcriptional activities of these proteins using HIV-1 LTR in various cells. Here we developed a novel protein transduction system that allows production of p27SJ and

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doi:10.1016/j.antiviral.2007.11.006

its excretion in culture media where it can be taken up by the other cells in a biologically active form, and suppress HIV-1 gene expression and replication in other cells containing the viral genome.

In a previous study, we demonstrated that expression of p27SJ results in suppression of HIV-1 promoter activity, partly due to its association and interference with the function of C/EBPβ, a cellular transcription factor that stimulates HIV-1 gene transcription. In an extension of those observations, we assessed the impact of p27SJ on the expression of the HIV-1 genome in microglial cells infected with HIV-1 JR-FL. Results from RNA analysis of the infected cells showed a substantial decrease in the level of full-length HIV-1 RNA in cells expressing p27SJ (Fig. 1A and B). The effect was specific, as p27SJ showed no detectable effect on the level of housekeeping RNA including GAPDH (Fig. 1C). The integrity of RNA preparation was tested by staining of the gel with ethidium bromide and observing the abundance of 18S and 28S RNAs (Fig. 1D). Expression of p27SJ had no major effect on the viability of primary cultures of microglial cells as tested by MTT and Glo assays (Fig. 1E and F).

These results along with our earlier observations prompted us to develop a novel protein transduction system that allows production of p27SJ and its efficient excretion in the media where it can be taken up, in a biologically active form, by the other cells and exert its inhibitory effect on HIV-1 gene expression. Toward this end, we constructed an expression vector in which p27SJ in fusion with the gene encoding green fluorescence protein (GFP) was flanked by an excretion signal (exc) derived from murine Ig-kappa chain signal peptide (Coloma et al., 1992) and an uptake signal (upt) from HIV-1 Tat (Ruben et al., 1989; Vives et al., 1997) (Fig. 2A). Two additional plasmids expressing GFP-p27SJ in fusion with either exc or upt singals were created and utilized as controls in our study. First, a plasmid encompassing GFP-p27SJ or its variants was introduced into the human astroglial cell line, U-87MG, and expression of the protein was evaluated by fluorescence imaging and immunoblot assays. As seen in Fig. 2B, in accord with the previous results, GFP-p27 showed a cytoplasmic appearance. Similarly, $_{exc}$GFP-p27SJ$_{upt}$ fusion protein was mainly accumulated in the cytoplasm. Incubation of microglial cells with the conditioned media from the cells expressing $_{exc}$GFP-p27SJ$_{upt}$ showed that nearly 30% of the incubated cells had taken up the chimeric protein. As expected, the control protein, GFP-p27SJ$_{upt}$ was mainly detected in the nuclei whereas $_{exc}$GFP-p27SJ was localized in the cytoplasm. Results from Western blot analysis verified detection of protein bands corresponding to GFP-p27SJ, $_{exc}$GFP-p27SJ, GFP-p27SJ$_{upt}$ and $_{exc}$GFP-p27SJ$_{upt}$ (Fig. 2C, lanes 1–4). Examination of the culture media from U-87MG cells by immunoblot showed the detection of both $_{exc}$GFP-p27SJ and $_{exc}$GFP-p27SJ$_{upt}$, suggesting that in the presence of a secretory signal, p27SJ can be released into the media. Results from functional assay indicated that only the conditioned media from the cells expressing $_{exc}$GFP-p27SJ$_{upt}$, but not GFP-p27SJ or any other derivatives, has the ability to significantly decrease the level of transcription from HIV-1 LTR in primary cultures of microglial, astrocytic and U-937 cell lines (Fig. 2D).

In our previous studies, we demonstrated that expression of p27SJ affects subcellular localization of C/EBPβ and results in

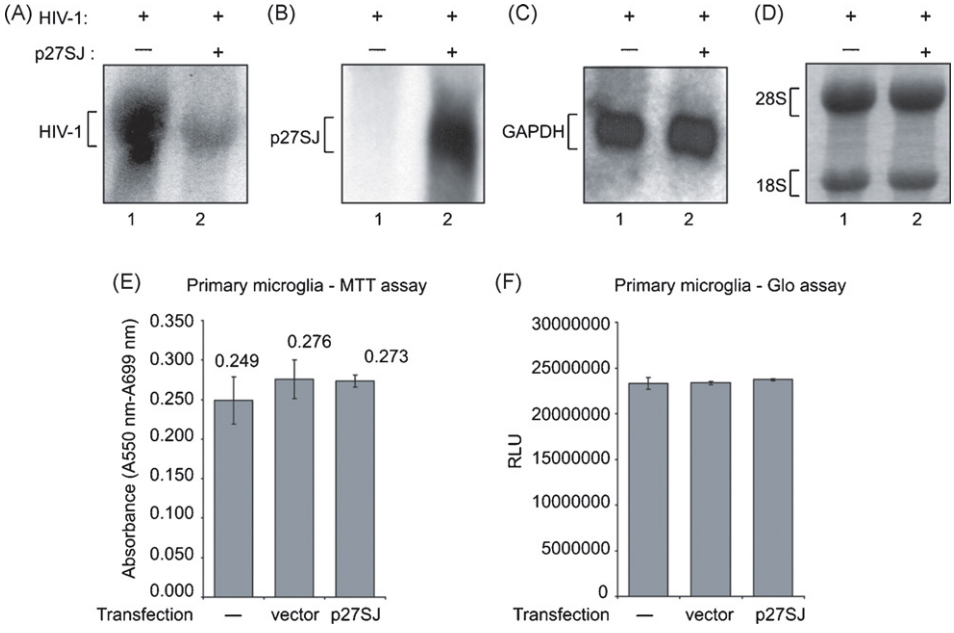

Fig. 1. Suppression of HIV-1 replication by endogenously expressed p27SJ. Primary culture of human microglial cells was transfected with CMV-p27SJ followed by infection with HIV-1 JR-FL. After 5 days, total RNA from HIV-1 infected cells, transfected and untransfected with CMV-p27SJ, was prepared and analyzed by Northern blot using DNA probes derived from the HIV-1 genome (A), p27SJ (B) and housekeeping GAPDH (C). The integrity of the RNA preparation was tested by staining of the gel with ethidium bromide and the position of the 18S and 28S RNAs as shown (D). Cell viability assays demonstrating cell proliferation/toxicity of p27SJ in primary culture of microglial cells, transfected with CMV-p27SJ or control empty vector, pCDNA6-B. After 48 h, cells were harvested and assayed by MTT (E) and GLO assays (F).

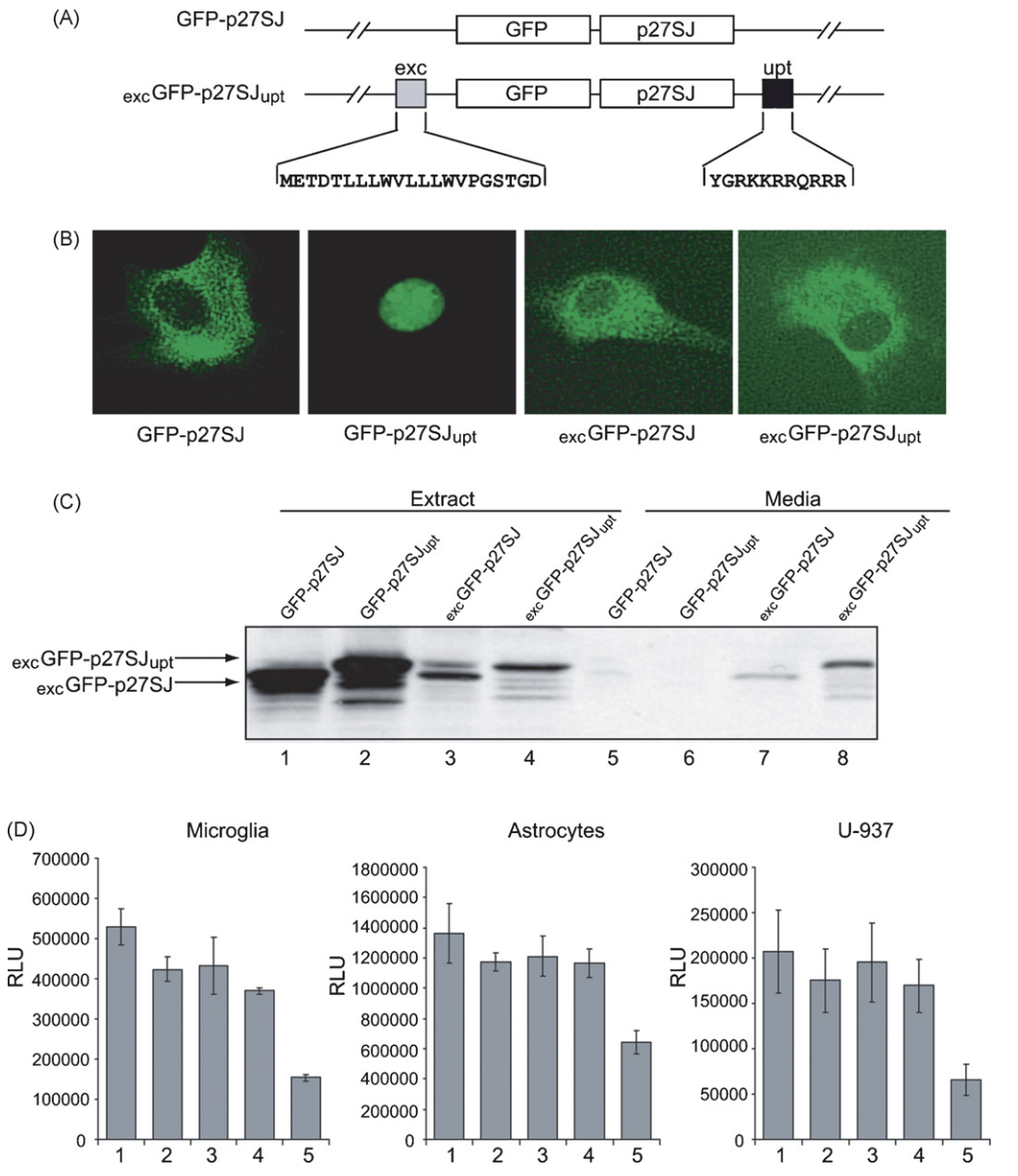

Fig. 2. Development of p27SJ protein transduction system. (A) Schematic illustration of the retroviral expression vector containing GFP-p27SJ or $_{exc}$GFP-p27SJ$_{upt}$ sequences. The DNA sequence corresponding to p27SJ was cloned in fusion with the DNA sequence encoding GFP and flanked by a 21 amino acid leader peptide from murine Ig-kappa light chain (excretion signal) and 11 amino acid arginine-rich protein transduction domain (PTD) from HIV-1 Tat (uptake signal). (B) Subcellular localization of p27SJ in U-87MG cells at 36 h after transfection with GFP-p27SJ and its various derivatives. (C) Immunoblot analysis of protein extracts from cells expressing GFP-p27SJ (lane 1), $_{exc}$GFP-p27SJ$_{upt}$ (lane 4), and the control fusion proteins (lanes 2 and 3), and the conditioned media (50 μl) from cells expressing $_{exc}$GFP-p27SJ (lane 7), $_{exc}$GFP-p27SJ$_{upt}$ (lane 8), and the control proteins. (D) Suppression of HIV-1 LTR activity by $_{exc}$GFP-p27SJ$_{upt}$ in primary culture of microglial, astrocytic and U-937 cell lines after transfection with LTR-luciferase reporter construct and incubation with conditioned media from cells expressing GFP-p27SJ, $_{exc}$GFP-p27SJ$_{upt}$ or the two control proteins.

cytoplasmic distribution of this transcription factor that is commonly found in the nucleus (Darbinian-Sarkissian et al., 2006). Thus, in the next series of studies, we investigated the subcellular localization of C/EBPβ in cells treated with media obtained from cells expressing $_{exc}$GFP-p27SJ$_{upt}$, and determined the ability of the imported protein to affect C/EBPβ stimulation of the HIV-1 LTR. Fig. 3A (left panel) shows DAPI staining of cells after treatment with cultured media containing $_{exc}$GFP-p27SJ$_{upt}$. As seen, only the cells that have taken up the protein, as evidenced by green fluorescence, exhibit cytoplasmic appearance of C/EBPβ, which is co-localized with $_{exc}$GFP-p27SJ$_{upt}$. Conversely, in cells with no uptake of $_{exc}$GFP-p27SJ$_{upt}$, C/EBPβ remained in the nucleus. Accordingly, results from the transcription studies showed that the level of C/EBPβ-induced transcription of the HIV-1 LTR is drastically decreased in primary cultures of microglia, astrocytes and U-937 cells after their treatment with $_{exc}$GFP-p27SJ$_{upt}$ (Fig. 3B).

In addition to its effect on C/EBPβ activity, p27SJ has the ability to interact with Tat protein and alter its function. Our earlier results showed that expression of p27SJ impedes the ability

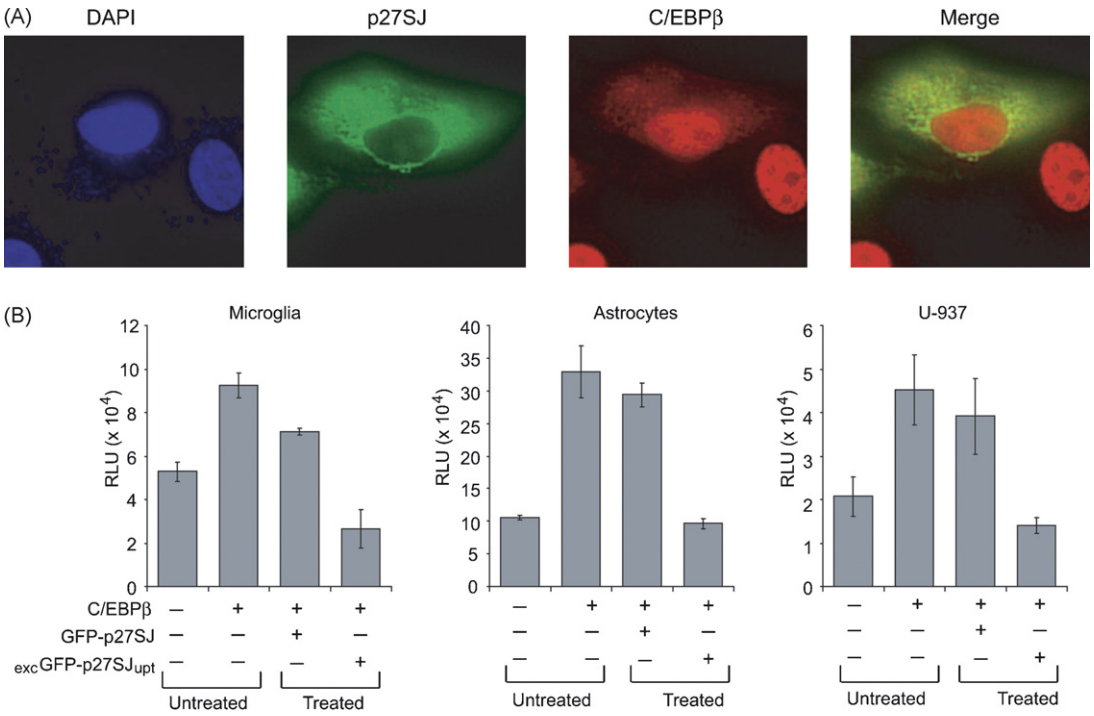

Fig. 3. Effect of p27SJ protein transduction system on C/EBPβ. (A) Immunocytochemistry of cells after treatment with conditined media containing $_{exc}$GFP-p27SJ$_{upt}$ using anti-p27SJ, anti-C/EBPβ or both, and DAPI for nuclear staining in U-87MG cells. (B) Incubation of primary cultures of microglia, astrocytic cell line and the human monocytic cell line (U-937) with conditioned media obtained from cells expressing GFP-p27SJ or $_{exc}$GFP-p27SJ$_{upt}$ after transfection with LTR-luciferase reporter plasmid in the presence of 2 μg of CMV-C/EBPβ expression plasmid.

of Tat to stimulate transcription of the HIV-1 LTR (Darbinian-Sarkissian et al., 2006). Here we demonstrate that treatment of primary cultures of human astrocytes and U-937 cells with culture media derived from cells expressing $_{exc}$GFP-p27SJ$_{upt}$ results in a substantial decrease in the level of Tat-induced activation of the HIV-1 LTR (Fig. 4A), suggesting that both basal and Tat-mediated expression of the LTR can be negatively controlled by exogenous p27SJ, which is armed by excreting and uptake signals.

Finally, we examined the ability of the secreted GFP-p27SJ in controlling HIV-1 replication. As shown in Fig. 4B, treatment of both U-937 and SupT cells with $_{exc}$GFP-p27SJ$_{upt}$ caused a nearly 50% decrease in the level of viral replication compared to the control cells that were treated with GFP-p27SJ.

p27SJ has a peculiar characteristic that allows its interaction with regulatory proteins including C/EBPβ, a DNA binding transcription factor that promotes expression of the HIV-1 LTR as well as the viral transactivator Tat, and suppresses transcription of HIV-1 and its replication in promonocytic cells and T-cells. By arming p27SJ at the N-terminus with secretion signals derived from immunoglobulin light chain, and placing at the C-terminus, an uptake signal from HIV-1 Tat, we created a new bi-directional protein transduction system that facilitates intercellular transport of the protein in a biologically active form. Our choice for selection of the signal from Ig light chain was based on earlier studies demonstrating the ability of this protein to be released by the cells as part of assembled antibody molecules and also as a free peptide (Coloma et al., 1992; Dul et al., 1996; Leitzgen et al., 1997). Our initial results showed that fusion of the 21 amino acid peptide of the Ig light chain to GFP resulted in excretion of this protein from the cells and its accumulation in the culture media (data not shown). Inclusion of the protein transduction domain (PTD) of Tat in the C-terminus of GFP showed no detectable effect on the excretion of the protein, yet permitted its efficient uptake by the treated cells. PTD of Tat has been utilized by several laboratories including our own to deliver various proteins that possess the ability to modulate cell growth (Chen et al., 1999; Darbinian et al., 2001; Merilainen et al., 2005; Schwarze et al., 1999; Viehl et al., 2005; Vocero-Akbani et al., 1999). For example, proteins that modulate tumor cell growth and promote cell death such as p53 (Kanwal et al., 2004), p16 (Gius et al., 1999), p21 (Hu et al., 2007; Massodi et al., 2005), p27 (Nagahara et al., 1998), Bcl-2 (Cao et al., 2002), merlin (Bashour et al., 2002), and others have been cargoed by Tat to cancer cells in culture or in animal models. Our study is among the few studies that utilized the PTD of Tat for delivery of potential inhibitors of HIV-1 into cells. Our approach is unique as we utilize both excretion and uptake signals to increase the efficiency of exporting of the peptide by the donor cells and facilitate its import by the recipient (target) cells. In this respect, p27SJ appears to be a suitable protein as it exerts its inhibitory action on both the basal level of HIV-1 expression and on Tat-induced activation of the viral genome. Our results also show that expression of the engineered p27SJ has no toxicity upon the donor and recipient cells. Thus, it may serve as a potential therapeutic agent that can be utilized either alone or in combination with other treatments for suppressing HIV-1 replication.

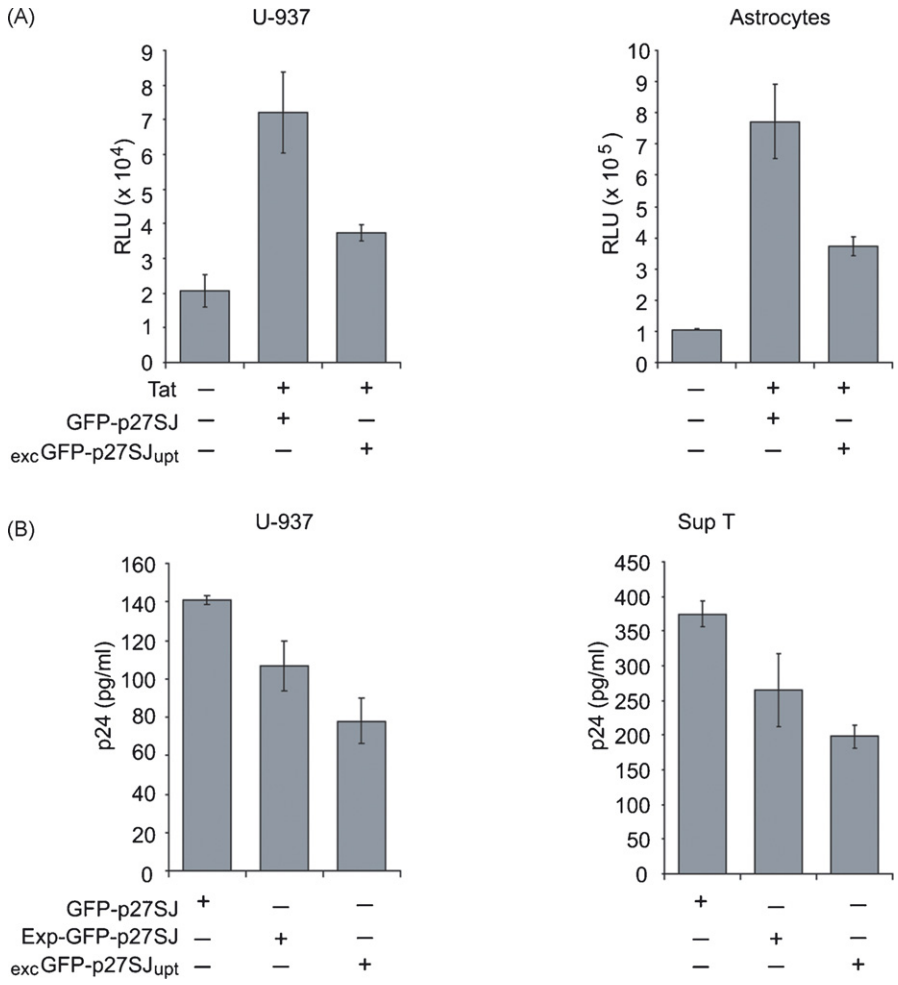

Fig. 4. Suppression of LTR activity and inhibition of HIV-1 replication by secreted p27SJ protein. (A) Treatment of human promonocytic cells, U-937, and astrocytes with conditioned media obtained from cells expressing GFP-p27 or $_{exc}$GFP-p27SJ$_{upt}$ after transfection with LTR-luciferase reporter plasmid in the presence of CMV-Tat. (B) Approximately 2×10^6 U-937 cells and T-lymphocytic SupT cells grown in RPMI with 2% fetal bovine serum were infected with HIV-1 JF-RL and treated with conditioned media obtained from cells expressing GFP-p27, $_{exc}$GFP-p27SJ or $_{exc}$GFP-p27SJ$_{upt}$. Five days post-infection, supernatants were collected and analyzed for the presence of p24 by ELISA assay.

Acknowledgements

We thank past and present members of the Department of Neuroscience particularly Marta Czernik, Kenneth Simbiri, Dr. Sujatha Radhakrishnan, Alla Arzumanyan, Dr. Armine Darbinyan and Dr. Bassel Sawaya for their technical and scientific input on this work. We also thank C. Schriver for preparation of this manuscript. This work was supported by grants awarded by NIH to SA.

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