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A novel role for Slit2/Robo1 axis in modulating HIV-1 replication in T cells

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Abstract

Objective—Slit2 is a secreted glycoprotein that has been shown to possess anti-inflammatory properties. In addition, Slit2 has been shown to modulate CXCR4-mediated functional effects in T cells. However, its role in HIV-1 pathogenesis is not known. The objective of the current study is to analyze the role of Slit2 in modulating HIV-1 replication in T cells.

Methods—The effect of endogenous Slit2 expression on HIV-1 replication in T cells was studied by transient overexpression or downregulation of Slit2. The role of exogenous Slit2 was studied by analyzing the effect of soluble Slit2 protein on HIV-1 replication in T-cell lines and peripheral blood mononuclear cells (PBMCs).

Results—Studies on T-cell lines revealed a higher expression of Slit2 in Jurkat T cells compared with MT4 cells. We observed that downregulation of Slit2 in Jurkat T cells using Slit2-specific small inhibitor RNA enhanced HIV-1 replication. However, overexpression of Slit2 in MT4 cells and PBMCs reduced HIV-1 replication. As Slit2 is a secretory protein, we further analyzed the role of soluble Slit2 on HIV-1 virus replication using various cell lines and PBMCs. Our data indicated that exogenous Slit2 inhibited replication of both X4-tropic and R5-tropic HIV-1 viruses. Further studies revealed that Slit2 mediated its functional effects by binding to Robo1 receptor.

Conclusion—Taken together, our results describe Slit2/Robo1 axis as a novel modulator of HIV-1 replication in T cells. These studies may contribute to the understanding of the immunopathogenesis of HIV-1 infection.

Keywords

HIV-1 inhibition; Robo1; Slit2; T cells

Introduction

HIV-1 infects and replicates in CD4⁺ T cells and in cells of the monocyte/macrophage lineage using CD4 molecule and the chemokine receptors, CXCR4 or CCR5, to penetrate

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Conflict of interest

The authors have no conflicting financial interests.

into host cells [1]. A variety of cytokines and endogenous soluble factors have been shown to modulate HIV-1 replication during the course of the disease [2-5]. Slits are a group of large secreted glycoproteins originally described in regulating neural migration [6,7]. Slit consists of a family of three genes: *Slit1*, *Slit2* and *Slit3*, all of which are expressed in the nervous system. However, *Slit2* and *Slit3* can also be found in several nonneuronal tissues. Robo1, the predominant receptor for *Slit2* belongs to a novel subfamily of immunoglobulin superfamily proteins [8,9] and is also expressed in several nonneuronal tissues, including leukocytes. However, information on the effects of *Slit2*/Robo1 axis in nonneuronal systems is still fragmentary, with recent studies indicating its role in multiple processes including tumor growth and metastasis [10-14]. With regard to the immune system, *Slit2* was shown to decrease inflammation and disease progression in animal models of global ischemia [15], crescentic glomerulonephritis [16] and sepsis [17]. *Slit2* has been shown to inhibit migration of various immune cells toward chemoattractant signals [18-22]. Specifically, we and others have recently shown *Slit2* blocks CXCL12/ CXCR4-mediated functional effects in T cells [22,23]. The chemokine receptors, CXCR4, along with CCR5, are important coreceptors for HIV-1 virus.

In the present study, we analyzed the role of *Slit2* in HIV-1 replication. We demonstrate, for the first time, that endogenously expressed *Slit2* in T cells modulates HIV-1 replication. Furthermore, we demonstrate that soluble *Slit2* upon binding to Robo1 receptor modulates both X4-tropic and R5-tropic HIV-1 replication in T-cell lines and peripheral blood mononuclear cells (PBMCs).

Methods

Anti-HIV-1 assays

PBMCs were isolated from heparinized venous blood collected from healthy HIV-seronegative donors (American Red Cross, Columbus, Ohio, USA) as described before [20]. The viral isolates, HIV-1 IIIB, BaL, JRFL, HxBc2 and NL4-3, were obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program.

The T-cell line, MT4, was pretreated with various concentrations of *Slit2* and infected with HIV-1 IIIB (p24 10 ng per 10⁶ cells), washed extensively after 2 h of infection to remove the residual virus and incubated for 48 h. Phytohemagglutinin (PHA)-stimulated PBMCs pretreated with *Slit2* were infected similarly with HIV-1. However, PBMCs [in interleukin (IL-2)-containing complete Roswell Park Memorial Institute (RPMI)] were incubated for up to 2 weeks. Every 3–4 days, half the cell suspension was removed and replaced with fresh medium containing the same concentration of *Slit2*. HIV-1 p24 antigen levels in the culture supernatant were assessed by ELISA (Advanced Bioscience Laboratories, Rockville, Maryland, USA).

Immunostaining and flow cytometry

To determine intracellular p24 expression, HIV-1-infected T cells were fixed and permeabilized using Fix/perm solution (BD Biosciences, San Diego, California, USA) and stained with KC57 monoclonal antibody (Coulter, Brea, California, USA). Robo1 receptor

was analyzed by flow cytometry using Robo-1 antibodies (Abcam, Cambridge, Massachusetts, USA) or control antibody, as described previously [20].

Soluble Slit2 production

HEK293 cells that produce full-length myc-tagged Slit2 proteins have been described [22]. Slit2 and vector control-conditioned media were concentrated and partially purified according to procedures described previously [20]. For our initial studies, we used partially purified conditioned media from Slit2/myc-transfected HEK293 cells at a concentration of approximately 6 – 15 nmol/l. The concentration of Slit2 in the conditioned medium was estimated based on silver staining of serial dilutions of supernatants following SDS-PAGE fractionation (<http://links.lww.com/QAD/A176>). Conditioned medium from vector-transfected HEK293 cells at similar amounts were used as controls. These concentrations of Slit2-conditioned media have been described in various studies to analyze the functional effects of Slit2 [19-21,24].

For further studies, Slit2 in the conditioned medium was purified, as described previously [20]. The fractions were analyzed on 8% SDS-PAGE gels, stained with silver stain or on immunoblots and probed with anti-myc antibodies. The fractions containing Slit2 were dialyzed with PBS, concentrated and used for the HIV-1 replication assays.

Transfections

The pSecTagB-Slit2 construct [12] or control pSecTagB vector were transfected onto T-cell lines and primary cells using Nucleofector V or T solution (Amaxa Biosystems, Cologne, Germany) according to the manufacturer's instructions. Small inhibitor RNA (siRNA)-mediated knockdown of Robo-1 and Slit2 was performed using specific ON-TARGETplus SMARTpool siRNA (Dharmacon Lafayette, Colorado, USA.) by nucleofection with 200 nmol/l siRNA. The respective, nontargeted siRNA was used as a control.

Western blot analysis

Cell lysates were subjected to western blot analysis, as previously described [20].

Statistical analysis

Reported data are the means $\pm$ SD of at least three independent experiments performed in duplicate or triplicate. The statistical significance was determined by the Student's *t*-test.

Results

Endogenous Slit2 regulates HIV-1 replication in T cells

We have previously shown that Slit2 modulates CXCR4-mediated functional effects in T cells [20]. As CXCR4 also acts as a coreceptor for HIV-1, we investigated the role of Slit2 in modulating HIV-1 replication. Analysis of Slit2 expression in Jurkat T cells and MT4 cells revealed that Jurkat T cells expressed higher amount of Slit2 compared with MT4 cells (Fig. 1a, left panel). We also demonstrated Slit2 expression in PBMCs from normal donors (Fig. 1a, right panel). Next, we examined the effect of downregulation of endogenous Slit2 in Jurkat T cells using Slit2-specific siRNA on HIV-1 replication (Fig. 1b, left panel). We

observed that downregulation of constitutive Slit2 enhanced HIV-1 replication in Jurkat T cells by approximately 1.6-fold (Fig. 1b, right panel).

To further confirm the role of Slit2 in HIV infection, we overexpressed Slit2 in MT4 cells and PBMCs by transfecting pSecTagB-Slit2 construct [12] or pSecTagB control vector in these cells. The efficiency of the transfection was confirmed by determining Slit2 expression in cell lysates by western blot analysis (Fig. 1c, left top panel). We also observed Slit2 protein in the supernatants of T cells after Slit2 overexpression (Fig. 1c, bottom panel), indicating that overexpression results in Slit2 secretion. The Slit2-transfected MT4 cells showed approximately two-fold inhibition of HIV-1 replication (Fig. 1c, right panel), whereas the Slit2-transfected PBMCs showed an inhibition of approximately 3.5-fold using HIV-1 IIIB (Fig. 1d, left panel) and approximately two-fold using HIV-1 BaL (Fig. 1d, right panel) in comparison with vector-transfected cells, suggesting that overexpression of Slit2 in T cells was protective against HIV-1 infection. Together, these studies indicate that endogenously expressed Slit2 in T cells may play an important role in regulating HIV-1 infection.

Soluble Slit2 suppresses both X4-tropic and R5-tropic HIV-1 replication

As Slit2 is a secretory protein, we further determined whether soluble Slit2 would block HIV replication. Initially, we evaluated the effect of soluble Slit2 on HIV-1 replication using partially purified Slit2-conditioned medium or equal amounts of vector control-conditioned medium. Slit2 expression was detected in the conditioned medium by western blotting with anti-myc (Fig. 2a, left panel) or anti-Slit2 antibody (data not shown). Slit2-conditioned medium showed approximately five-fold inhibition of viral replication in MT4 cells at the end of 48 h as compared with the control (Fig. 2a, middle panel). We next showed that Slit2-conditioned medium also resulted in approximately two-fold inhibition of HIV-1 IIIB replication in PBMCs at 7–14 days in comparison to the control (Fig. 2a, right panel). Furthermore, we determined the anti-HIV activity of a highly purified form of Slit2 (Fig. 2b, left panel) prepared according to our published studies [23]. Purified Slit2 showed a dose-dependent inhibition of HIV-1 IIIB replication in MT4 cells with approximately five-fold reduction in HIV-1 replication at 6-nmol/l Slit2 (Fig. 2b, middle panel). Purified Slit2 also inhibited HIV-1 IIIB replication in PBMCs, with an inhibition of approximately three-fold to four-fold at 4–14 days (Fig. 2b, right panel). In addition, we evaluated other HIV-1 X4 isolates, HxBc2 and NL4-3, and found similar inhibition of HIV-1 replication (data not shown).

We next addressed the question whether Slit2 also modulates replication of R5-tropic strains. We observed that Slit2-conditioned medium (Fig. 2c, left panel) or purified Slit2 in a dose-dependent manner (Fig. 2c, right panel) inhibited HIV-1 BaL viral replication in PBMCs. Our findings were also confirmed using other R5-tropic strains including JRFL and YU2 (data not shown). Furthermore, we also confirmed the anti-HIV activity of both the Slit2-conditioned supernatant and purified Slit2 on CD4⁺ T cells isolated from PBMCs (<http://links.lww.com/QAD/A176>). In all experiments, we found no significant difference in cell growth and viability in uninfected cells in the presence and absence of Slit2 (<http://>

[links.lww.com/QAD/A176](https://doi.org/10.1186/1745-6216-17-176)). The above results, thus, confirm that Slit2 can inhibit HIV-1 replication independent of viral tropism and of the cell type used to produce virus.

Anti-HIV activity of Slit2 is mediated through Robo1

Slit2 has been shown to mediate its effects by binding to Robo1 receptor [6,8,9,20,22,24-30]. We found that Robo1 is expressed on MT4, Jurkat T cells and PBMCs using western blot analysis and flow cytometry (data not shown). Next, we determined whether Slit2-mediated anti-HIV effects are mediated through Robo1. MT4 cells transfected with Robo1 siRNA showed a significant knockdown of Robo1 expression as compared with nontargeted siRNA transfected cells (Fig. 2d, left panel). As shown in Fig. 2d, right panel, Robo1 downregulation abrogated Slit2-mediated inhibitory effects compared with control, suggesting that the anti-HIV activity of Slit2 is predominantly transduced by Robo1.

Discussion

Slit2 has recently been shown to modulate CXCR4-mediated functional effects and possess anti-inflammatory properties. However, nothing is known about its role in HIV pathogenesis. In the present study, we provide the evidence, for the first time, linking Slit2 to modulation of HIV-1 replication, thus adding a new dimension to the biological functions to Slit2.

We demonstrated Slit2 expression in both T-cell lines and PBMCs. The expression of endogenous Slit2 and its function in leukocytes has not been studied previously. Our studies indicate that Slit2 overexpression inhibited HIV-1 replication in MT4 and PBMCs, whereas inhibition of constitutive Slit2 expression in Jurkat T cells slightly enhanced HIV-1 replication, suggesting that endogenous Slit2 expression and secretion may modulate HIV-1 replication in T cells probably by acting in an autocrine/paracrine manner. We also showed that soluble Slit2 inhibited both X4-tropic and R5-tropic HIV-1 using both partially purified Slit2-conditioned medium and purified Slit2. Several factors expressed by host cells, including APOBEC3G/F, TRIM5 α , CD317 and Murr1 [31-34] as well as soluble mediators such as IL-10, IL-27 and type 1 interferon, have also been shown to inhibit HIV-1 replication [2-5]. We propose that Slit2 could be a novel soluble factor expressed by host cells that may modulate HIV-1 replication. Our finding that Slit2 may modulate HIV-1 replication by binding to Robo1 receptor is in agreement with various recent studies that have shown that Slit2 binds to Robo1 to mediate many of its functional effects [35-37]. Our preliminary studies indicated that Slit2 did not affect HIV receptor/coreceptor expression in T cells (data not shown). We have previously shown that Slit2 modulates CXCL12/CXCR4 signaling by inhibiting Akt and Rac1 activation [20], both of which have been shown to play an important role in mediating HIV entry [38,39]. We, therefore, hypothesize that Slit2 may inhibit HIV by modulating signaling pathways that mediate viral entry.

Conclusion

Overall, our studies demonstrate a novel role for Slit2/Robo1 axis in HIV replication and may contribute to the understanding of HIV-1 pathogenesis.

Acknowledgments

A.R.A. designed and performed research, analyzed data and prepared the manuscript; T.N. designed, performed research and analyzed data; and R.K.G. designed the research, analyzed data and prepared the manuscript.

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Fig. 1. Overexpression and downregulation of Slit2 modulates HIV-1 replication

(a) Slit2 expression in lysates from MT4 cells and Jurkat T cells loaded at different concentrations (left panel) and from peripheral blood mononuclear cells (PBMCs) isolated from three normal donors (right panel) was determined by western blot (WB) analysis. Equal protein loading was determined by probing the same blot with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody. (b) Slit2 expression in Jurkat T cells transfected with nontargeting (NT) or Slit2-specific small inhibitor RNA (siRNA) was analyzed by WB analysis (left panel). Intracellular p24 staining of the transfected cells infected with HIV-1 IIIB for 48 h was determined by flow cytometric analysis (right panel). * $P < 0.05$ versus the NT-siRNA-transfected cells. (c) Slit2 expression in MT4 cells transfected with either control vector (pSecTagB) or pSecTagB-Slit2 construct was determined by WB analysis (left upper panel). Equal protein loading was confirmed using GAPDH antibody (left middle panel). Slit2 expression in the culture supernatants was analyzed by WB analysis (left lower panel). Intracellular p24 staining of the transfected cells

infected with HIV-1 IIIB for 24h was determined by flow cytometric analysis (right panel). * $P < 0.05$ versus the vector-transfected cells (VCs). (d) Intracellular p24 staining of PHA-activated PBMCs from normal donors transfected with control vector (pSecTagB) or pSecTagB-Slit2 and infected with HIV-1 IIIB (left panel) or HIV-1 BaL(right panel) for 48 h was determined by flow cytometric analysis. * $P < 0.05$ versus the VCs. Data represent mean $\pm$ SD of three independent experiments.

Fig. 2. Exogenous Slit2 inhibits HIV-1 replication in T cells by binding to Robo1 receptor
 (a) Slit2 expression in partially purified conditioned media from Slit2-myc-transfected human embryonic kidney (HEK) cells (Slit2) or vector-transfected cells (VCs) was determined by WB analysis (left panel). HIV-1 IIIB replication in MT4 cells (middle panel) or PHA-stimulated peripheral blood mononuclear cells (PBMCs) from normal donors (right panel) in the presence of partially purified conditioned medium containing Slit2 (approximately 6 nmol/l) or conditioned medium from control HEK293 cells was determined by analyzing HIV-1 p24 antigen levels in the supernatants by ELISA. In the case of MT4 cells, the supernatants were collected after 48 h. Virus production in the positive control (HIV-1-infected MT4 cells cultured only with medium): 12.1 ng/ml p24 Ag. * $P < 0.05$ versus the mock-infected cells treated with control supernatant. PBMCs were incubated up to 2 weeks and supernatants were collected at various time points. Virus production in positive control (HIV-1 infected PBMCs cultured with medium only): 3 days,

0.45ng/ml; 7 days, 1.25 ng/ml; 10 days, 7.89 ng/ml; and 14 days, 15.69 ng/ml p24 Ag. * $P < 0.05$ versus the mock-infected cells treated with control supernatant. (b) Silver staining (SS) and WB analysis of Slit2 purified by fast protein liquid chromatography (left panel). HIV-1 IIIB replication in MT4 cells pretreated with various concentrations of purified Slit2 (middle panel) or PBMCs pretreated with purified Slit2 (6 nmol/l) (right panel) was determined by analyzing HIV-1 p24 antigen levels in the supernatants by ELISA. Virus production in the positive control (HIV-1 infected MT4 cells cultured only with medium): 10.3 ng/ml p24 Ag. * $P < 0.05$ versus the mock-infected cells. Virus production in the positive control (HIV-1 infected PBMCs cultured only with medium): 4 days, 0.5 ng/ml; 7 days, 3.4 ng/ml; 10 days, 8.89 ng/ml; and 14 days, 13.69 ng/ml p24 Ag. * $P < 0.05$ versus the mock-infected cells. (c) HIV-1 BaL replication in PHA-stimulated PBMCs in the presence of conditioned medium containing Slit2 (approximately 6 nmol/l) or control (left panel) or various concentrations of purified Slit2 (right panel) was determined by measuring HIV-1 p24 levels in the culture supernatants collected at various time points by ELISA. Virus production in the positive control (HIV-1-infected PBMCs cultured only with medium): 3 days, 0.7 ng/ml; 7 days, 2.9 ng/ml; 10 days, 7.6 ng/ml; and 14 days, 12.9 ng/ml p24 Ag. * $P < 0.05$ versus the mock-infected cells treated with control supernatant. Virus production in the positive control (HIV-1-infected PBMCs cultured only with medium): 4 days, 0.91 ng/ml; 7 days, 3.2 ng/ml; and 10 days, 8.7 ng/ml p24 Ag. * $P < 0.05$ versus the mock-infected cells. (d) Robo 1 expression in MT4 cells transfected with nontargeting (NT) or Robo small inhibitor RNA (siRNA) by nucleofection (Amaxa Biosystems) was determined by flow cytometry. HIV-1 IIIB replication in the NT and Robo1 siRNA-transfected cells pretreated with conditioned medium containing Slit2 (approximately 6 nmol/l) or control was analyzed by measuring HIV-1 p24 levels in the supernatants by ELISA (right panel). Virus production in the positive control (HIV-1-infected MT4 cells cultured only with medium): 11.7 ng/ml p24 Ag. * $P < 0.05$ versus the mock-infected cells transfected with NT-siRNA. Data represent mean $\pm$ SD of three independent experiments.