Human Immunodeficiency Virus Bearing a Disrupted Central DNA Flap Is Pathogenic In Vivo

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Received 29 January 2007/Accepted 18 March 2007

The central DNA flap is an important component of lentiviral vectors, but its significance in the context of wild-type human immunodeficiency virus (HIV) is currently unclear. To address this issue, we have compared the in vitro infection kinetics of NL4-3 with those of a flap-deficient mutant and evaluated the in vivo growth characteristics of these viruses by using the SCID-hu mouse model of HIV infection. Flap-deficient virus was only modestly attenuated in vitro, as assessed by single-round and spreading infection assays, and exhibited levels of replication and pathogenesis close to those of the wild-type in vivo. Hence, an intact central flap is not essential for HIV replication.

All lentiviruses, including human immunodeficiency virus (HIV), have the capacity to productively infect nondividing cells (20, 22, 28, 39, 42). However, the specific mechanisms responsible for translocating the HIV preintegration complex through an interphasic nuclear membrane have yet to be fully elucidated (reviewed in references 10 and 43). One component of the HIV preintegration complex with a suggested role in nuclear import is a triple-stranded DNA structure termed the “central DNA flap” (44). The central flap is formed during reverse transcription, when approximately 99 nucleotides between the central polypurine tract (cPPT) and the central termination sequence are displaced during plus-strand DNA synthesis (13–15, 44). The addition of sequences encompassing the central flap to lentiviral vectors usually enhances their infectivity by approximately 2- to 10-fold or more (19, 31, 36, 41). Nevertheless, the level of enhancement bestowed by the central flap may differ substantially between that of lentiviral vectors and that of wild-type (Wt) HIV. This issue has been investigated by several groups, leading to conflicting reports in the literature regarding the requirement for the central DNA flap in viral replication. Some reports asserted that the central DNA flap was either essential (44) or very important (5, 16) for productive HIV infection, but others suggest a much more minor role in virus replication (29, 18). Hence, while the central flap is a highly conserved genetic element, its importance during Wt HIV infection remains uncertain. Furthermore, the contribution of the central flap to HIV replication and pathogenesis within an in vivo system has not previously been described.

To address these issues, we first constructed a flap-deficient (F−) mutant of Wt HIV, NL4-3 (1), by modifying 10 nucleotides in the cPPT and central U box (23) by using site-directed mutagenesis (Fig. 1A) as previously described (18, 29, 44). In order to accurately quantify HIV expression in single-round infection assays, a new NL4-3-based reporter virus was also constructed (Fig. 1B). This virus expresses the enhanced green fluorescent protein (EGFP) (12, 38) fused to firefly luciferase (17) (EGFPLuc) in place of HIV Env but maintains the integrity of all other HIV open reading frames and important cis-acting RNA elements. The proviral plasmid was generated as previously described for NL4-3-GFP (37), except that the primers 5′-ATTGGGTAACCAAAGGCCGCGTACCGGTGCCACCATG-26 and 5′-TTCGCAGGCAGTTACAGCTC GTCCCTCACGCCGACATTCGGCGCTC were used to amplify the EGFPLuc coding sequence by PCR using pEGFP-PLuc (Clontech) as the template. The cPPT-D modifications were introduced into this denv(Wt) vector to produce a corresponding denv(F−) reporter virus genome. Resultant plasmids were each cotransfected into 293FT cells (Invitrogen) with a vesicular stomatitis virus G glycoprotein-expressing vector to generate pseudotype viruses (11) for use in single-round infection assays. All subsequent in vitro infections were independently performed at least twice.

We established the functionality of these new reporter viruses by infecting 293FT cells. 293FT cells (105) were infected with denv(Wt) or denv(F−) viruses (6.25 pg of p24/infection) and visualized for GFP expression at 3 days postinfection. GFP expression was absent from mock-infected cells, but abundant GFP was observed for cells infected with each reporter virus (Fig. 1C). A Luc activity time course was also conducted, and increasing levels of Luc activity were detected in infected wells (Fig. 1D).

We then tested the Wt and F− viruses with single-round and spreading infection assays using transformed CD4+ T-cell lines. CEM cells (5 × 104) were infected with denv(Wt) or denv(F−) pseudotype viruses (50 ng of p24/infection), and cells were harvested at various times for quantification
of Luc activity. Very similar expression kinetics were evident for the two viruses (Fig. 2A). When GFP expression was assessed by flow cytometry at 3 days postinfection, a slightly higher percentage of GFP-positive (GFP⁺) cells was present in denv(Wt) than in denv(F⁻) infections (3.35% versus 2.2%, respectively), but the mean fluorescent intensity of GFP⁺ cells was comparable for each virus (Fig. 2B). Spreading infection characteristics for Wt and F⁻ viruses in CEM cells were also established. CEM cells (10⁵) were infected with Wt or F⁻ replication-competent viruses (10 ng of p24/infection), and aliquots of supernatant were assayed for p24 concentration (Fig. 2C). The F⁻ virus showed slightly delayed replication kinetics (approximately 1 day slower than those of Wt) but was not severely debilitated. Single-round and spreading infection assays performed in the same way using PM1 (30) target cells yielded similar results (Fig. 2D and E).

The different viruses were then compared using primary target cells. First, CD4⁺ T cells were isolated by negative selection from peripheral blood leukocytes, using immunomagnetic beads (CD4⁺ T-cell isolation kit II; Miltenyi Biotech). Cells were costimulated for 2 days and then subjected to both single-round (Fig. 3A) and spreading infection (Fig. 3B) assays as described above for CEM cells. The differences between Wt and F⁻ virus infections with these primary target cells were greater than those for transformed T-cell lines, but expression levels were still quite robust for the mutant viruses. A spreading infection assay using 2 × 10⁷ primary fetal thymocyte target cells (prepared as previously described in reference 27) and 100 ng of p24 for each virus showed a similar, modest reduction in replication capacity with the F⁻ virus (Fig. 3C).

It was possible that the central flap would have had a more profound effect upon HIV spread and pathogenesis in vivo than was evident during in vitro assays. The SCID-hu thymus/liver (Thy/Liv) mouse model (32, 35) of HIV infection is an extremely versatile in vivo system for characterizing HIV replication efficiency and pathogenesis (2, 7, 34). This model has been successfully utilized for comparing different HIV strains (6, 9, 25, 26), as well as accessory gene mutants (3, 4, 24) and viruses harboring mutations in other regions of the HIV genome such as the Rev response element (40). Hence, this model is particularly well suited for testing the in vivo effects of disrupting the HIV central DNA flap.

Thy/Liv implants within SCID-hu mice were mock infected or inoculated with equivalent amounts (10 ng of p24) of Wt or F⁻ viruses, and then wedge biopsies of implants were done at
week 3 postinfection, and the same mice were sacrificed and reassessed at week 5 postinfection, as previously described (3, 8). Levels of HIV replication within the tissue samples were determined by real-time PCR using primers designed to detect the R/U5 regions of the viral long terminal repeat (21). A PCR assay for human β-globin sequences was performed in parallel to standardize DNA input (Fig. 4A). Sequencing of several week-5 DNA samples (Fig. 4A and B) from F⁻ infected implants confirmed that the cPPT-D mutations had not reverted during the infections (data not shown).

Thymocytes were stained for a flow cytometry assay, and CD4/CD8 profiles were used to assess the pathogenic potential of each virus, as defined by their ability to deplete CD4⁺/CD8⁺ double-positive cells (Fig. 4C). The F⁻ virus replicated with an efficiency level close to that of the Wt (Fig. 4A) and caused depletion of CD4⁺/CD8⁺ thymocytes.
in a manner similar to that of the Wt but with slightly delayed kinetics (Fig. 4B).

The results described here demonstrate that the central flap can modestly enhance HIV replication in vitro and in vivo, which may explain its evolutionarily conserved nature. However, virus bearing a disrupted central flap was quite capable of initiating and maintaining a robust infection in all cell types tested and replicated efficiently in vivo, showing only slightly

FIG. 4. In vivo replication and pathogenesis of mutant and Wt viruses. Implants of SCID-hu (Thy/Liv) mice were mock infected or inoculated with Wt or F' replication-competent viruses and assessed at week 3 and week 5 postinfection. (A) Real-time PCR assays for HIV DNA and human β-globin DNA were performed using samples obtained at each time point. Values from all samples are shown individually. Gray icons represent those samples where sequencing was performed to verify maintenance of the cPPT-D mutations throughout infections. Black icons represent the samples where detailed flow cytometry scatter plots are shown in panel C. Differences between viral loads of mock-infected and infected samples are significant (P < 0.001) at both time points. The viral loads in tissues infected with Wt virus are significantly higher than those of tissues infected with the F' mutant at week 3 (P < 0.01) but not at week 5 (P > 0.2). (B) CD45^+ (human) cells were analyzed for CD4^+CD8^- profiles by flow cytometry to assess depletion of CD4^+ CD8^- cells by the different viruses. Values are expressed as the percentage of CD4^+ CD8^- double-positive cells present in each implant. Mock values are significantly higher than those of infected samples at both time points (P < 0.001). Percentage values for the F' virus are significantly higher than those for Wt at both week 3 (P < 0.05) and week 5 (P < 0.02). (C) Scatter plots showing flow cytometry profiles of samples at, or closest to, the median percentage of CD4^+ CD8^- cells within each group. All statistical comparisons were performed using the Wilcoxon rank-sum test (two sided). A second, independent experiment using different human donor tissue to generate the SCID-hu (Thy/Liv) mice yielded very similar results to those presented here (data not shown).
REFERENCES


