

A Single Amino Acid Substitution in the HIV-1 gp41 Cytoplasmic Domain Affects Assembly of a Patient-Derived Envelope Glycoprotein

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Abstract

Background: The incorporation of envelope glycoprotein onto budding virions is an important step during assembly of Human Immunodeficiency Virus type 1 (HIV-1) in infected cells. The cytoplasmic domain of envelope protein (gp41) has been shown to carry determinants important in envelope incorporation onto virus particles. Two related envelope genes amplified by PCR from lymph node tissue of an AIDS patient conferred cell:cell fusion but were inefficiently assembled onto virions. Here we have analyzed these envelopes for their assembly defects.

Methods: The envelope genes were subcloned into an envelope expression vector (pSVIIIenv) and pseudotype virus was produced by co-transfection of pSVIIIenv and pNL4.3env^v in 293T cells. Specific point mutations were introduced in the envelope gene by PCR. Single round infectivity assays were done on CD4⁺CCR5⁺ GHOST cells. Cell-cell fusion assays were done by mixing env^v-293T cells and target cells, e.g., GHOST/CCR5 cells. Envelope incorporation onto virions was assessed by Western blot of filtered and pelleted virions.

Results: The patient envelopes expressed on 293T cells induced cell:cell fusion of CD4-CCR5⁺ cells. Pseudotype virions harvested from 293T cells cotransfected with patient env^vpSVIIIenv and pNL4.3env^v conferred only very low levels of infectivity on CD4⁺CCR5⁺ GHOST cells. The low infectivity correlated with minimal envelope incorporation onto virions as determined by Western blot. Sequencing and mutagenesis analyses showed critical determinants mapped to a single R787H substitution in a region of the gp41 cytoplasmic domain, previously implicated in gag:env interactions. Reversion of the substitution restored envelope assembly onto virions and infectivity. However, when the same substitution (R787H) was introduced into the NL4.3 envelope, no effect on assembly or infectivity was observed.

Conclusion: These results indicate that R787 in the gp41 cytoplasmic domain is an important determinant in envelope assembly but can be complemented by unknown determinant/s present in the NL4.3 envelope.

Introduction

The incorporation of envelope glycoproteins (gp160) onto budding virions is an important step during assembly of the human immunodeficiency virus type 1 (HIV-1). The cytoplasmic domain of the envelope protein (gp41) has been shown to carry determinants important for envelope incorporation and infectivity. Five related envelope (gp160) clones were amplified from a patient (NA420); three from brain (B13, B33 and B42) and two from lymph nodes (LN40 and LN85). In contrast to the NA420 brain envelopes, the LN envelopes conferred very low infectivity and were inefficiently assembled onto virions. Here, we have analyzed these envelopes to identify determinants in the gp41 cytoplasmic domain, responsible for assembly defects.

Summary

1. NA420 LN envelopes were inefficiently assembled onto virus particles. The infectivity of lymph node envelopes were also severely compromised compared to NA420 brain envelopes.
2. NA420 lymph node envelopes were expressed and functional in cell-cell fusion assays.
3. Sequence analyses revealed five changes in the gp41 cytoplasmic regions between NA420 LN40 and NA420 B33.
4. Mapping studies identified a single R→H change at position 787 in the gp41 cytoplasmic domain responsible for the loss of assembly for NA420 LN40 envelope.
5. In NL4.3, substitution of R787H did not confer loss of envelope assembly onto virions and is thus compensated by unknown determinants.

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Isolation and characterization of envelope genes from AIDS patient.

5 envelope genes were amplified from patient NA420 brain and lymph node autopsy tissue samples as described by Peters *et al.* (1). Table 1 summarizes the characteristic features of each envelope gene. NA420 brain envelopes were macrophage tropic, while those obtained from lymph node tissue were not.

Table 1. Patient envelope information

| Envelope ID | Source | Receptors |
|-------------|--------------------|-----------|
| NA420 B13 | Brain/Frontal Lobe | CCR5 |
| NA420 B33 | Brain/Frontal Lobe | CCR5 |
| NA420 B42 | Brain/Frontal Lobe | CCR5 |
| NA420 LN40 | Lymph node | CCR5/CCR3 |
| NA420 LN85 | Lymph node | CCR5/CCR3 |

Envelope expression and production of Pseudotype virus

Envelope genes amplified by PCR were subcloned into pSVIIIenv expression vector between KpnI sites. Pseudotype viruses were made by cotransfection of pSVIIIenv containing envelope of choice and env^vpNL4.3 (Figure 1) into 293T cells. Pseudovirions were harvested at 48 hours and reverse transcriptase activity (RT) measured by ELISA.

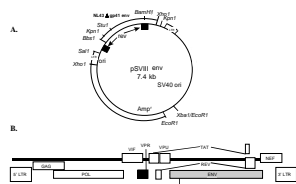


Figure 1. Vectors used to express envelopes and prepare pseudotype virus. A. pSVIIIenv envelope expression vector and **B.** pNL4.3 backbone with premature stop codon in env gene.

Envelopes amplified from patient NA420 Lymph Node confer low infectivity.

Five envelope genes amplified from subject NA420 were tested for their capacity to confer infectivity. Pseudovirions produced by cotransfection of 293T cells with pSVIIIenv containing NA420 envelopes and env^vpNL4.3 were tested on CD4⁺CCR5⁺GHOST or NP2 glioma cells in a single round replication assay. As shown in Figure 2, lymph node derived envelopes conferred significantly less infectivity (17-32 fold) compared to from brain tissue. Since LN envelopes amplified from other patients conferred high infectivity (not shown), we concluded that NA420 LN envelopes were defective.

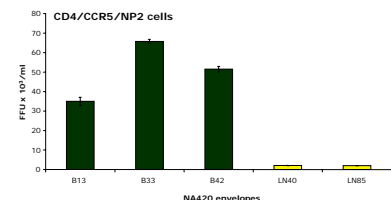


Figure 2. Infectivity conferred by patient NA420 envelopes. Infectivity was tested on CD4⁺CCR5⁺NP2 cells. Cells were fixed 72 hours after infection and immunostained for p24. Infectivity titers were then scored as focus forming units (FFU) per milliliter.

NA420 Envelopes derived from lymph node are expressed and functional

We next tested whether NA420 LN40 and NA420 LN85 envelopes were expressed and fusing. 293T cells were transfected and permeabilized 48 hours later with methanol:acetone (1:1), before immunostaining using Cheshire 8, an anti-gp41 Mab. Positively stained, envelope-expressing cells were clearly visible for each mutant envelope construct (Figure 3A).

Figure 2B shows syncytia induced following cocultivation transfected env^v 293T cells with GHOST/CD4/CXC4 cells. Both NA420LN40 and NA420LN85 envelope were functional for cell:cell fusion and induced comparable levels of syncytia to NA420B33 envelope.

Results

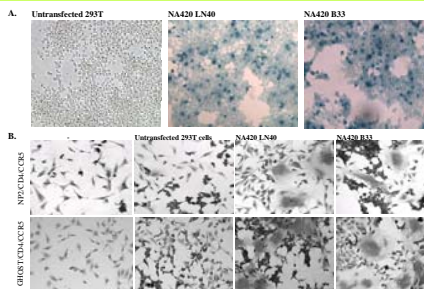


Figure 3. Envelope expression and function. A. 293T cells transfected with env^vpNL4.3 and pSVIIIenv containing patient NA420 brain and lymph node envelopes were immunostained for envelope. Cells were fixed and permeabilized with methanol:acetone and immunostained with anti-gp41 mab, Cheshire 8. **B.** Cell-cell fusion induced by NA420 mutant envelopes. Env^v 293T cells were cocultivated overnight with NP2/CD4/CCR5 and GHOST/CD4/CCR5 cells and were fixed and stained with 1% methylene blue, 0.25% basic fuchsin in methanol.

Defective NA420 LN envelope assembly onto virions correlates with low infectivity

We next investigated whether the NA420 LN40 envelopes were assembled onto virions. The amount of envelope assembled onto virus particles for NA420 LN40 was measured and compared with NA420 B33. Env^v pseudotype viruses produced from 293T cells were harvested, filtered through 0.45µm syringe filter and pelleted at 100,000 g for 2 hours. Each env^v virus preparation were resolved by SDS-PAGE, blotted and probed for gp41. As shown in Figure 4, the amount of NA420 LN40 on virions was drastically reduced compared to NA420 B33 envelope. Thus, a severe decrease of virion-associated envelope likely explains their low infectivity.

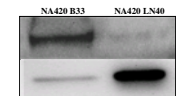


Figure 4. Incorporation of NA420 envelopes onto virus particles. 293T cells were co-transfected with env^vNL4.3 and pSVIIIenv containing NA420 B33 and NA420 LN40 envelopes. Supernatants harvested 48 hours after transfection were spun at low speed and filtered through a 0.45µm syringe filter. The supernatants were then ultracentrifuged at 100,000 g for 2 hours at 4°C to pellet virus particles, which were resolved by SDS-PAGE followed by Western blot using anti-gp41, Cheshire 8 or anti-p24, 183-H12-5C as primary antibodies.

Sequence comparison between NA420 B33 and NA420 LN40

The gp41 sequences were compared between NA420 LN40 and NA420 B33. We specifically looked at differences in the gp41 cytoplasmic region, known to contain residues important for envelope incorporation and infectivity (2). Five residue differences were noticed in the gp41 domains between NA420 LN40 and NA420 B33. This included a C→F substitution at position 764 in LN40 that eliminates the linkage site for palmitic acid, a moiety involved in envelope association with lipid rafts and assembly onto virions. A pair of substitutions RR→HS were also noted at positions 787 and 788 respectively. Experiments were therefore designed to map and identify determinants in the gp41 cytoplasmic domain that caused the differences in infectivity between NA420 brain and lymph node envelopes.

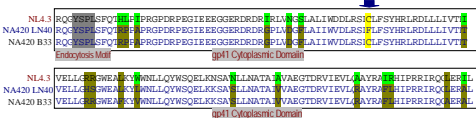


Figure 5. Sequence differences in gp41 cytoplasmic regions. The envelope sequences in the gp41 cytoplasmic region of NL4.3, NA420 LN40 and NA420 B33 envelopes were aligned using MacVector™7.2.2 program. The differences in residues between the envelope sequences were highlighted.

Mapping of determinants in NA420 LN40 envelope conferring low infectivity and envelope assembly onto virions.

To map the determinants in NA420 LN40 that abrogate envelope assembly, the gp41 fragments between NA420 LN40 and NA420 B33 were swapped and point mutations generated by PCR. Figure 6A shows the amino acid variants of gp41 generated. Pseudotype viruses were generated by transfection of 293T cells and infectivity assessed on GHOST CD4/CCR5 cells. As shown in Figure 6B, substitution of histidine and serine at positions 787 and 788 for arginines (present in B33) restored infectivity. Restoration of palmitoylated cysteine at position 764 had little effect on infectivity. Single substitutions in the LN40 HS motif were also tested. Thus, H787R restored infectivity up to ~70% compared to NA420 B33. As expected H787R also restored envelope incorporation onto virions (Figure 6D). Thus, R787 is a key determinant in gp41 cytoplasmic domain that is important for assembly and infectivity. The level of GFP expression in GHOST/CD4/CCR5 cells as a measure of HIV-1 assembly also highlights the differences in phenotype between NA420 LN40 (F-HR) and other variants (Figure 6C).

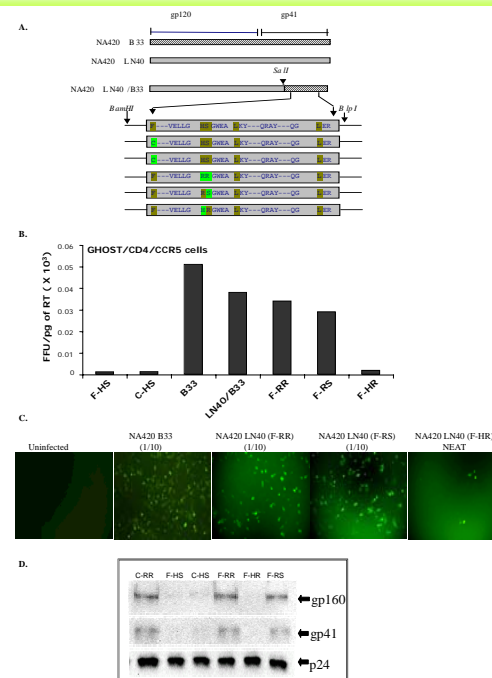


Figure 6. Mapping of critical determinants that modulate infectivity. A. Amino acid substitutions introduced in the gp41 cytoplasmic domain of NA420 LN40 by PCR mutagenesis. **B.** Infectivity was tested on CD4⁺CCR5⁺ indicator cell lines. Infected cells were fixed and immunostained for p24. Infectivity titers were then scored as focus forming units (FFU) per picogram of RT in virus supernatants as measured by ELISA. **C.** Infectivity was also assessed by GFP expression under a fluorescent microscope. **D.** Incorporation of NA420 LN40 mutant envelopes onto virus particles were assessed by SDS-PAGE followed by Western blotting as described in Figure 4.

The R787H substitution is tolerated in NL4.3 envelope

We further tested whether R787H substitution in NL4.3 gp41 cytoplasmic domain results in similar phenotype as NA420 LN40. Point mutations were carried out by PCR to introduce R787H and R788 in NL4.3 envelope. Progeny pseudovirions produced from 293T cells were tested for infection on GHOST/CD4/CXC4 cells and directly compared with NA420 LN40. As shown in Figure 7, substitutions of HS at positions 787 and 788 respectively in the NL4.3 envelope did not confer loss of envelope infectivity (7A) or assembly (7B). These observations suggest that an unknown determinant in NL4.3 must compensate for the loss of arginines (R787H and R788S).

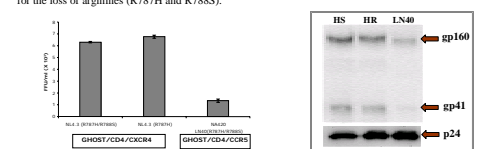


Figure 7. Infectivity conferred by HIV-1 NL4.3 envelopes lacking RR787/788 motifs in gp41 cytoplasmic domain. A. Infectivity was tested on GHOST/CD4/CXC4 (NL4.3) and GHOST/CD4/CCR5 (NA420 LN40). Cells were fixed 72 hours after infection and immunostained for p24. Infectivity titers were then scored as focus forming units (FFU) per picogram of RT in virus supernatants as measured by ELISA. **B.** Envelope incorporation was assessed by SDS-PAGE followed by Western blot using Cheshire 8 and 183-H12-5C to probe envelope and p24.

References

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