The effect of the membrane-proximal tyrosine-based sorting signal of HIV-1 gp41 on viral infectivity depends on sequences within gp120

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Abstract

The cytoplasmic domain of the HIV-1 Env glycoprotein (gp41) contains sequences that affect the trafficking of Env within the host cell. We previously showed that the membrane-proximal tyrosine-based adaptor protein (AP)-binding signal of gp41 (Y712XXL) is required for optimal viral infectivity and entry into target cells. Because these effects were not attributable to an effect on the incorporation of Env into virions, we hypothesized that they involved targeting of viral assembly to specific endosomal membranes that conferred greater fusogenicity. To further elaborate this hypothesis, we mutated the C-terminal leucine-based AP-binding signal of gp41 (LL855/856). In contrast to Env Y712, the leucine signal was dispensable for viral infectivity in both single cycle assays and during spreading infections within cultures of peripheral blood mononuclear cells (PBMCs). To test the hypothesis that these AP-binding motifs target Env to endosomes during viral morphogenesis, we compared the subcellular localization of wild-type Env to mutants of the Y712 and LL855/856 signals. The results failed to support the hypothesis that these signals target viral assembly to specific endosomal membranes. Strikingly, in the context of a C2-V3 region that confers macrophage-tropism, mutation of Y712 no longer markedly affected viral infectivity in either single cycle assays or during spreading infection within PBMCs, and it did not impair viral entry. These data indicate that the importance of the tyrosine-based sorting signal in gp41 for optimal viral infectivity depends on sequences in gp120. This observation is consistent with the hypothesis that the Y712 residue is part of the ectodomain of gp41 in virion-associated Env. We speculate that as part of the ectodomain, Y712 could affect specifically the conformation of the more positively charged CXCR4-tropic V3 loop in a manner that augments viral fusogenicity and infectivity.

Keywords: HIV-1; Envelope; gp41; gp120; Tyrosine sorting signal; Leucine sorting signal; Tropism; Cytoplasmic tail; Morphogenesis; Assembly

Introduction

The envelope (Env) glycoprotein of human immunodeficiency virus type 1 (HIV-1) is present on the surface of the virus particle and is essential for mediating viral entry into the target cell. The native structure of Env is trimeric and consists of three surface subunits (gp120) and three transmembrane subunits (gp41). During viral morphogenesis, HIV-1 virions bud from a cellular membrane at which the Env glycoproteins as well as the viral structural proteins and enzymes accumulate. HIV-1 is thought to bud primarily from the plasma membrane of T lymphocytes; however, in primary macrophages virions also form within intracellular structures such as late endosomes or multivesicular bodies (Nguyen et al., 2003; Ono and Freed, 2004; Pelchen-Matthews et al., 2003; Raposo et al., 2002). In HeLa cells, the majority of Env is also located at internal membranes rather than at the plasma membrane (Blot et al., 2003; Day et al., 2004; Otteken et al., 1996; Wyss et al., 2001). It seems likely that HIV-1 Env is transported to intracellular compartments for a purpose, but the sorting pathways utilized by Env and their impact on determining the site of virion morphogenesis are not well understood.

The cytoplasmic tail of the transmembrane subunit of Env contains sequences that have been identified as sorting signals; these signals affect the trafficking and targeting of Env within a
host cell. A membrane-proximal tyrosine-based sorting signal (Y712XXL) in the cytoplasmic tail of gp41-Env interacts with the clathrin adaptor AP-2 and mediates the endocytosis of Env from the plasma membrane (Bertoloz-Torrent et al., 1999; Boge et al., 1998; Ohno et al., 1997; Rowell et al., 1995). This tyrosine-based signal in Env has also been shown to be important for the polarized release of virions from a cap-like structure in lymphocytes and from the basolateral membranes of polarized epithelial cells (Deschambeault et al., 1999; Labranche et al., 1995; Lodge et al., 1994, 1997). A leucine-based sorting signal (LL855/856) at the distal end of the gp41 cytoplasmic tail binds the clathrin adaptor AP-1 (Wyss et al., 2001). When the leucines were mutated to alanine, Env maintained a prominent juxtanuclear localization in HeLa cells, but it became less well distributed to the periphery of the cytoplasm (Wyss et al., 2001). However, the importance of the LL855/856 signal to viral infectivity and replication kinetics has not been reported. In addition to the AP-binding signals, a diaromatic motif (YW802/803) in the cytoplasmic tail of gp41 has been described to mediate the trafficking of Env by binding to TIP47, a protein required for retrograde transport of proteins from endosomes to the trans-Golgi network (Blot et al., 2003). In HeLa cells, wild-type Env concentrated in a juxtanuclear region coinciding with markers of the TGN, whereas Env containing a mutated YW signal was dispersed in vesicles throughout the cytoplasm. The YW signal was also reported to be essential for the production of infectious virus and the incorporation of Env into virions (Blot et al., 2003).

We previously showed that the gp41 EnvY712XXL sequence of NL4-3 is important for the production of maximally infectious virus (Day et al., 2004). Viral propagation in primary lymphocytes, single-round infectivity, and viral entry into target cells were impaired when the Y712 was mutated to alanine. Virion-incorporation of gp120 was slightly reduced by this mutation when virions were produced from a T lymphoblastoid cell line (MT4); however, no significant decrease in the incorporation of gp120 was detected in virions produced from fibroblastoid cells (293T). The infectivity and entry defects of EnvY712A virus, despite little to no differences in the incorporation of gp120 into virions, suggested that another mechanism enables this motif to maximize viral infectivity (Day et al., 2004).

We hypothesized that this mechanism involves AP-mediated trafficking of Env and the targeting of viral assembly to specific cellular membranes such as endosomes. As noted above, studies of viral morphogenesis in primary macrophages suggest that virions can bud on endosomal membranes, specifically CD63-positive late endosomes or multivesicular bodies. It seems conceivable that a specific composition of proteins, phospholipids or cholesterol present at the late endosomal membrane might enable virions assembled there to be more fusogenic than those assembled at the plasma membrane.

Consequently, we tested the hypothesis that Y712 of gp41 targets Env to specific endosomal membranes. We also studied the importance of the C-terminal leucine-based AP-binding signal for viral infectivity and replication. Although we found no evidence to support specific endosomal targeting mediated by these motifs, we discovered that in the context of a macrophage-tropic C2-V3 region, the Y712 signal was no longer required for optimal viral infectivity and replication. We also observed that the macrophage-tropic envelope glycoprotein increased the single-round infectivity of wild-type virions by six-fold in comparison to the parental CXCR4-tropic clone. This increase in infectivity correlated with increased viral entry into target cells and an increased amount of gp120 within virions. Based on these results, we propose a novel alternative hypothesis for the mechanism underlying the specific importance of the Y712 residue for viral infectivity and entry in the context of CXCR4-tropism.

**Results**

EnvY712 is required for efficient viral infectivity and replication whereas LL855/856 is not

We previously showed that the Y712XXL signal of Env is important for viral infectivity and entry into target cells, but the importance of the EnvLL855/856 signal remained unknown (Day et al., 2004). To study the affect of mutation of the leucine-based sorting signal, the two Env leucines within the proviral construct pNL4-3 were changed to alanines using site-directed mutagenesis. Viruses were produced by transient transfection of 293T cells and quantified by ELISA analysis of the p24 capsid antigen. First, the infectivities of NL4-3 wild-type, EnvY712A and EnvLL855/856AA viruses were tested in a single-round infectious-center assay. Serial dilutions of each virus were used to infect HeLa-CD4 indicator cells (P4.R5), and the expression of β-galactosidase was detected by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The numbers of blue-stained, HIV-infected cells were counted using image analysis software developed in our laboratory (Day et al., in press). As expected, the Y712A mutation resulted in virus that was less infectious as compared to wild-type NL4-3 across the entire range of inocula (Fig. 1A). The number of infected cells per nanogram of p24 for EnvY712A ranged from 22% to 28% of the wild-type value. Analysis of the slopes of the linear portion of the infectivity curves (inocula below 50,000 pg of p24) indicated that EnvY712A was 23% as infectious as wild-type virus. In contrast, EnvLL855/856AA virus was nearly as infectious as wild-type NL4-3 (Fig. 1A). The number of infected cells per nanogram of p24 for EnvLL/AA ranged from 73-101% of wild-type across the range of inocula studied, and the slope of the infectivity curve was 73% of the wild-type value. These data indicated that the leucine-based signal in Env has relatively little affect on the infectivity of the NL4-3 virus.

To determine the importance of these Env sorting signals during replication in a natural target cell, the spread of wild-type and mutated viruses within cultures of activated peripheral blood mononuclear cells (PBMC) was measured. Equal amounts of each virus as normalized by content of p24 capsid antigen were used to infect phytohemagglutinin (PHA) activated PBMCs isolated from healthy blood donors. Viral propagation within the cultures was detected by measurement of p24 in the culture supernatant. Similar to the data from the single-cycle infectious-
center assay, EnvY712A virus was impaired in viral replication during the 10-day time-course, whereas EnvLL/AA grew virtually as well as wild-type NL4-3 (Fig. 1B). These data confirmed the importance of EnvY712 in viral infectivity and replication and indicated that the Env leucine-based signal was relatively unimportant.

Mutations in putative sorting signals have no apparent affect on the intracellular localization of Env

The mechanism underlying the importance of EnvY712 for viral infectivity is unknown. We previously showed that in viruses produced from CD4-negative 293T cells, the infectivity defect (and the defect in viral entry) of EnvY712A virus was not attributable to decreased incorporation of gp120 into virions. We hypothesized that an alternative mechanism for the effect of the Y712A sequence might involve AP-mediated trafficking of Env and the targeting of viral assembly to specific endosomal membranes.

To test this hypothesis, we first studied the subcellular localization of wild-type Env with the goal of identifying an endosomal marker that overlapped extensively with Env at steady state. HeLa-CD4 cells (P4.R5) were transiently transfected with wild-type NL4-3 provirus; in some experiments, the cells were co-transfected with plasmids expressing endosomal marker proteins fused to GFP. Proteins were localized by confocal immunofluorescence microscopy 24 h later. Surprisingly, we found little colocalization of wild-type gp41-Env with the endosomal markers we studied: Rab5-GFP, EEA1 (early endosomes); Rab7-GFP, CD63 (late endosomes); and Rab11-GFP (recycling endosomes) (Fig. 2). Although incomplete, overlap was detected between gp41 and a marker for the trans Golgi network (TGN46) (Fig. 2). Interestingly, the TGN as identified by this marker was dispersed in the virus-expressing cells.

Despite the lack of an endosomal marker that robustly co-localized with wild-type Env, we next examined the subcellular localization of the Env mutants. Neither the Y712A nor the LL855/856AA mutations altered the apparent distribution of gp41 within the cells (Fig. 3A). In addition, no marked differences in gp41 localization relative to TGN46 were detected (Fig. 3B). To ensure that the HeLa cells used for the morphologic analysis above supported the role of Y712A in viral infectivity, we measured the infectivity of viruses produced by these cells using the single-round infectious-center assay (Fig. 3C). These data revealed that these HeLa cells, like HEK 293T cells and primary lymphoblasts, support the phenotype of the Y712A mutant with respect to viral infectivity, despite the apparent lack of effect of this mutation on the subcellular distribution of gp41.

Because the majority of data suggesting endosomal assembly of HIV-1 have used primary macrophages as host cells, we introduced the Y712A and LL855/856AA mutations into a macrophage-tropic clone for morphologic study; for this purpose, we used an NL4-3-derivative containing the Env C2-V3 region from a primary isolate (51-9) (Chesebro et al., 1992). Primary monocyte-derived macrophages were infected with these viruses and stained for gp41. Again, no apparent differences were detected in the subcellular distribution of gp41 between wild-type, Y712A and LL/AA Env within these natural target cells of HIV-1, although we were also unable to confirm co-localization of wild-type Env with CD63 (data not shown). Taken together, these data failed to support the hypothesis that tyrosine-based or leucine-based sorting signals within the gp41 subunit of Env direct viral morphogenesis to specific endosomal membranes.

Mutation of EnvY712 within a macrophage-tropic virus does not markedly affect viral infectivity, replication, or entry

Unexpectedly, we observed that the macrophage-tropic virus, which presumably uses CCR5 for its co-receptor, did not support the virologic effects of the Y712A mutation as shown above in the context of CXCR4-tropic Env (Fig. 4). Notably,
Fig. 2. Wild-type gp41-Env shows little overlap with endosomal membrane markers. P4.R5 cells were transiently transfected using FuGENE 6 with full-length NL4-3 provirus, and in some experiments cells were co-transfected with GFP fusion proteins (see the Materials and methods). Proteins were localized by confocal immunofluorescence microscopy 24 h later. A single optical section is shown. Insets show a magnified region of the boxed area outlined in the merge image.
the parental CCR5-tropic clone, 51-9, was made previously by replacing the portion of gp120 that includes the C2 and V3 regions of NL4-3 with the same region from a primary HIV isolate (Chesebro et al., 1992). Using the infectious-center assay described above, we observed that the EnvY712A mutation reduced viral infectivity only slightly in the context
of the CCR5-tropism (81% of wild-type by slope-analysis across the entire range of inocula; Fig. 4A). Similar to CXCR4-tropic NL4-3, the EnvLL/AA mutant remained as infectious as wild-type (Fig. 4A). To determine whether EnvY712 was required in the context of 51-9 for maximal replication within cultures of primary cells, we infected activated PBMCs with equal amounts of virus normalized by p24 capsid and monitored viral output for 10 days. There was virtually no difference in viral growth between wild-type, Y712A and LL/AA Env viruses in this assay (Fig. 4B). These data indicated that unlike the CXCR4-tropic parental NL4-3, the CCR5-tropic derivative 51-9 did not support a defect in infectivity and replication caused by the EnvY712A mutation. Notably, the absolute infectivity (number of infectious units per nanogram of p24 inoculum) was six times higher for 51-9 than for NL4-3 and peak viral replication in PBMCs was reached two days earlier, indicating that 51-9 was a more infectious molecular clone (compare Figs. 1 and 4).

Previously, we showed that the impaired infectivity of EnvY712A virus was in part due to a defect in viral entry using a flow cytometry-based β-lactamase-Vpr (BlaM-Vpr) entry assay (Day et al., 2004). To determine whether the CCR5-tropic EnvY712A mutant virus lacked a defect in viral entry, we tested 51-9 wild-type and its isogenic Y712A Env mutant in the entry assay. We hypothesized that the Env Y712A mutation within 51-9 would no longer result in an entry defect, similar to the results of the infectivity assay. BlaM-Vpr containing viruses were produced by transient co-transfection of 293T cells with a full-length proviral plasmid encoding 51-9 and a second plasmid encoding the BlaM-Vpr fusion protein. Fusion of BlaM-Vpr containing virus with the plasma membrane of target cells (P4.R5) was detected using a fluorescent substrate that is specifically cleaved by β-lactamase, causing a shift in the emission wavelength from green to blue. The data were expressed as a ratio of blue to green fluorescence, with a high ratio indicative of viral entry. Whereas uninfected control cells that were loaded with the substrate showed a low level of blue fluorescence (blue-green ratio gated to score 1.6% of the uninfected cells as positive), cells infected with wild-type NL4-3 virus resulted in 8.3% entry-positive cells, five times more than background (Fig. 5). In contrast, only 3.3% of cells scored positive for viral entry when exposed to NL4-3 EnvY712A. These data are consistent with those published previously, in which the efficiency of viral entry by the EnvY712A mutant was half that of wild-type (Day et al., 2004). Interestingly, the entry of 51-9 virus was much more efficient than NL4-3, with 47% cells scoring positive for viral entry (Fig. 5). Furthermore, there was no apparent change in the efficiency of entry when Y712 of Env was mutated. The increased entry-efficiency of wild-type 51-9 compared to wild-type NL4-3 was consistent with its increased infectivity in the infectious-center assay presented above: six times as many cells scored entry-positive with 51-9 than with NL4-3. Together, these data indicated that this NL4-3 derivative containing a CCR5-tropic V3 loop was six times more infectious than the parental CXCR4-tropic virus. Furthermore, its infectivity, replication-rate, and efficiency of entry into target cells were unaffected by a change in the membrane proximal tyrosine-based sorting signal of gp41.

The macrophage-tropic virus, 51-9, contains a greater amount of virion-associated gp120 than the parental CXCR4-tropic virus, NL4-3

Because the 51-9 virus was six times more infectious than NL4-3 and did not support a defect caused by EnvY712A mutation, we characterized the levels of gp41 and gp120 incorporated into the viral membrane. Viruses were produced from 293T cells, filtered, pelleted by ultracentrifugation, and assayed by p24-ELISA before loading equal amounts of capsid antigen onto an SDS-PAGE gel (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Viral proteins were detected by Western blot using antibodies to gp120 and p24. As published previously, NL4-3 wild-type and Y712A Env viruses showed little or no difference in the incorporation of gp120 into virions (Fig. 6A) (Day et al., 2004). Similarly, 51-9 wild-type and EnvY712A virions showed little difference in gp120-incorporation. A lower exposure of the same gp120 blot confirmed little difference in gp120 intensity between 51-9 wild-
type and EnvY712A viruses (Fig. 6A, middle panel), and 3-fold dilutions of 51-9 virus probed for gp120 also confirmed this lack of difference (data not shown). However, relative to NL4-3, 51-9 virions contained a greater amount of gp120 (Fig. 6A). Using an antibody to gp41, the four viruses tested showed little difference in gp41 incorporation, suggesting that the difference in gp120 between NL4-3 and 51-9 was a result of differential shedding of gp120 (Fig. 6B). Lysates of the cells that produced the viruses tested were normalized for p24 capsid concentration, and then used to infect P4.R5 cells for 5 h. After washing, cells were incubated with the β-lactamase-sensitive fluorochrome CCF2/AM for 16 to 18 h at 25 °C. Analysis was performed by flow cytometry. Uncleaved substrate was detected as blue fluorescence. Data are expressed as a ratio of blue to green fluorescence and are gated using uninfected cells (control).

Discussion

Based upon previous data, we hypothesized that the Y712XXL AP-binding sequence in the cytoplasmic tail of gp41 is important for viral infectivity by directing virion assembly to specific endosomal membranes. We found no evidence to support this hypothesis using immunofluorescence microscopy. We also tested the hypothesis that the leucine-based AP-binding motif at the C-terminus of gp41 (EnvLL855/856) influenced viral infectivity. In contrast to mutation of Y712, which decreased viral infectivity in a single-cycle infectious-center assay and reduced the rate of viral replication in primary PBMCs, mutation of EnvLL855/856 had no effect. Unexpectedly, we discovered that in the context of a macrophage-tropic virus, which presumably uses CCR5 as its co-receptor, the Y712A mutation no longer markedly affected viral infectivity or replication-rate. Notably, the CCR5-tropic variant used, though differing from the parental NL4-3 only in the sequence of the C2-V3 region, was more infectious, more efficient at viral entry, and reached peak replication in PBMCs faster than CXCR4-tropic NL4-3. An increased amount of gp120 within virions likely accounted for the greater infectivity of this CCR5-tropic virus. Importantly, the failure of the Y712A mutation to cause a striking infectivity-defect in the context of the CCR5-tropic V3 loop was observed consistently over a broad range of concentration of input virus, weighing against an inoculum-effect as the reason for the loss of the Y712A infectivity-phenotype. Based on these results, we speculate that Y712 may specifically affect the conformation of the CXCR4-tropic V3 loop in a manner that augments viral infectivity.

The initial goal of these studies was to test the hypothesis that the AP-binding signals within gp41 target viral assembly to a specific endosomal compartment whose membrane-composition was optimal for viral fusogenicity. In general, the sorting signals within the cytoplasmic tail of HIV-1 Env have been
replication rate within cultures of PBMCs was confirmed, as defect of the Y712A mutant in single-round infectivity and Env within virions (Day et al., 2004). In the present study, although there was little or no difference in the incorporation of tyrosine to alanine or cysteine resulted in less infectious virus, West et al., 2002). We previously published that mutation of the proximal tyrosine-based sorting signal, substitution of the tyrosine (Y712) with serine or cysteine was reported to yield incorporation into virions (Blot et al., 2003). In contrast, within the membrane-associated gp120 than NL4-3. Virus-containing supernatants produced from transfected 293T cells were filtered through 0.2-μm-pore-size filters and centrifuged at 23,500 × g for 2 h at 4 °C. Viral pellets were then resuspended in a small volume of reducing SDS sample buffer, normalized for p24 concentration, electrophoresed on a reducing SDS-PAGE gel, and then electroblotted to membranes. (A) Proteins were stained using antibodies to gp120 (upper and middle panels) or p24 (lower panel). The middle panel represents a lower exposure of the same blot shown in the upper panel. (B) Proteins were stained using antibodies to gp41 (upper panel) or p24 (lower panel). The data presented are representative of 3 independent experiments. (C) Cell lysates of the virus-producing 293T cells were harvested at the time of virus harvest by resuspending cell pellets in reducing SDS-PAGE sample buffer. The lysates were normalized for p24 concentration, electrophoresed, and blotted as in panel A.

shown to affect viral infectivity to various degrees, but only in some cases have these effects correlated directly with changes in the virion-incorporation of Env. For example, an intact diatomic motif (YW802/803) was reported as necessary for the production of infectious virus and the incorporation of Env into virions (Blot et al., 2003). In contrast, within the membrane-proximal tyrosine-based sorting signal, substitution of the tyrosine (Y712) with serine or cysteine was reported to yield virions of increased infectivity relative to the wild-type, but changes in the incorporation of Env into virions did not correlate with the increases in infectivity (Cervantes-Acosta et al., 2001; West et al., 2002). We previously published that mutation of the tyrosine to alanine or cysteine resulted in less infectious virus, although there was little or no difference in the incorporation of Env within virions (Day et al., 2004). In the present study, the defect of the Y712A mutant in single-round infectivity and replication rate within cultures of PBMCs was confirmed, as was the lack of difference in the incorporation of Env into virions between wild-type and Y712A NL4-3 virus when produced from 293T cells. However, using immunofluorescence microscopy, we could find no evidence for changes in the intracellular localization of Env when the tyrosine signal was mutated. Consequently, we could not provide direct morphologic support for the hypothesis that the virologic role of this signal is based on the direction of viral assembly to specific endosomal membranes. Notably, we were unable to identify a cellular marker that extensively co-localized with Env in the HeLa cells used herein. Consequently, it remains possible that the endosomal-morphogenesis hypothesis is correct, but that the lack of a robust cellular marker for the sites of Env accumulation at steady-state precluded the detection of subtle differences in the localization of Y712A Env relative to wild-type.

To further test the endosomal-morphogenesis hypothesis, we studied another AP-binding motif in gp41, the leucine-based motif at the extreme C-terminus. This signal, LL885/886, was important neither for single-round infectivity nor replication-rate, despite its reported ability to direct the interaction of gp41 with the endosomal clathrin-adaptor, AP-1 (Wyss et al., 2001). Furthermore, we detected no apparent change in the subcellular distribution of this mutant relative to wild-type. Consequently, the analysis of this C-terminal LL-based signal did not support the putative role of endosomal targeting of Env in viral infectivity. Interestingly, this leucine-based signal has been proposed as a co-factor for the effect of Nef on viral infectivity (Schiavoni et al., 2004), a conclusion that seems unlikely in view of its lack of impact on viral infectivity as shown here.

If the Y712XXL signal influences neither the virion-incorporation of Env nor the sites of Env accumulation in the cytoplasm at steady-state, then how does it affect viral infectivity? Moreover, why does this signal lose its virologic effect in the context of a CCR5-tropic V3 loop? As noted above, although the substitution of a primary, macrophage-tropic C2-V3 region within NL4-3 yielded virus of markedly increased infectivity, we do not think it likely that this accounts for the loss of the Y712A phenotype, because we have measured single-round infectivity over a wide range of inocula. Even at the lowest concentrations of input virus (at which the effective multiplicity of infection was within the range of that achieved using the parental NL4-3; compare Figs. 1 and 4) the Y712A mutation only minimally decreased infectivity in the CCR5-tropic context. Instead, we propose an alternative explanation for the observation that the Y712A mutation encodes a V3 loop-dependent virologic phenotype: it may directly affect the conformation of the gp120 subunit.

How might the Y712A mutation affect the conformation of gp120 in a V3-loop dependent manner? One possibility is that Y712 affects either the relationship between gp41 trimers or the conformation of the ectodomain of gp41 in a manner that in turn affects the conformation of gp120. In support of this, changes within the cytoplasmic tail of gp41 have been shown to affect the association between gp41 and gp120 (Affranchino and Gonzalez, 2005). In a manner similar to cell surface receptors that transmit signals to their cytoplasmic domains, the conformation and function of gp120 may be influenced by the conformation of the cytoplasmic tail of gp41. To render the 51-9
virus insensitive to the effects of Y712, substitution of the C2-V3 region of NL4-3 gp120 with a CCR5-tropic sequence may confer greater conformational stability upon gp120 such that mutation of Y712 no longer negatively affects virus infectivity.

In addition to the possibility that Y712 within the cytoplasmic tail of gp41 affects indirectly the conformation and function of gp120, Y712 could actually be part of the ectodomain in virion-associated Env, where it directly affects the conformation of CXCR4-tropic Env in a manner that enhances viral infectivity. In support of this possibility, Dimmock and colleagues have proposed that gp41 exists in two alternative conformations, one containing the traditional single membrane-spanning domain (MSD) and a second containing three membrane-spanning domains (Cheung et al., 2005; Dimmock, 2005; Heap et al., 2005; Hollier and Dimmock, 2005). In the proposed 3-MSD-structure, a 40-residue loop of the cytoplasmic tail resides on the outer surface of the viral membrane, creating a previously undefined minor ectodomain of gp41; this loop includes the Y712XXL sequence. The 3-MSD conformation of gp41 creates the possibility that Y712 directly interacts with gp120, and it provides a potential mechanism by which the effect of the Y712A mutation can be relatively specific to virus containing a CXCR4-tropic V3 loop. Specifically, the CXCR4-tropic V3 loop of NL4-3 has a +4 charge relative to the CCR5-tropic V3 loop of 51-9 and an overall +5 charge within the entire C2-V3 loop. Consequently, it is conceivable that if present in the viral membrane, CXCR4-tropic V3 loop. Y712 could actually be part of the ectodomain, Y712 might interact preferentially with the more positively charged CXCR4-tropic V3 loop and play a more important role in viral infectivity in this context (Gallivan and Dougherty, 1999; Waters, 2004). Whether through a direct interaction with gp120 or through a conformational change transmitted from the cytoplasmic tail, Y712 of gp41 affects viral infectivity in a C2-V3 dependent manner.

Finally, as noted above, we observed that the CCR5-tropic virus 51-9 was more infectious, more efficient at entry, and replicated faster within PBMCs than the parental virus, NL4-3. We observed by Western blot that 51-9 virions contained more gp120 than NL4-3; this likely accounts for the increased infectivity and rate of replication of this virus. The data further suggested that the greater gp120 content of 51-9 virions was due to less gp120 shedding. The observation that CXCR4-tropic Env is more susceptible to shedding from virions has been made previously. Lab-adapted strains of HIV that predominantly use CXCR4 have been observed to experience greater sensitivity to soluble CD4 and greater gp120 shedding than primary isolates that typically use CCR5 (O’Brien et al., 1994). Chimeric viruses similar to that used in the present study were used to show that the V3 loop determined the relatively greater sensitivity of CXCR4-tropic viruses to inhibition by soluble CD4 (Lundquist et al., 2004). Interestingly, we observed a striking increase in the gp120 content of virions mediated by the substitution of a CCR5-tropic C2-V3 sequence for a CXCR4-tropic sequence, even though the viral producer cells were CD4-negative. We also observed evidence suggesting more efficient processing of gp160 in the case of the Env containing the macrophage-tropic C2-V3 sequence.

In summary, although our studies do not disprove the hypothesis that Y712XXL-mediated endosomal-trafficking of Env plays a role in viral infectivity, they provide no direct morphologic support for this model. Strikingly, and potentially inconsistent with an endosomal-trafficking model, the importance of the Y712 residue to infectivity appears to depend on sequences within gp120. The mechanism of this phenomenon is open to speculation. We are intrigued by the hypothesis of a 3 membrane-spanning domain conformation of gp41 and its potential to allow a direct interaction between Y712 and the relatively positively charged CXCR4-tropic V3 loop. Developing an effective method to distinguish between these potential alternate conformations of gp41 within infected cells and virions may facilitate an understanding of the mechanism behind the virologic role of the Env Y712XXL sequence. Finally, it is important to note that Y712 is extremely well conserved throughout HIV-1 sequences, regardless of the predicted tropism of the V3 loop (data not shown). This conservation could reflect the seemingly minor but consistently detected role of this residue in virion-infectivity in the context of CCR5-tropism (Fig. 4), or it may reflect potential roles of this motif in virologic effects not directly measured here, such as polarized budding and cell-cell transmission (Deschambeault et al., 1999; Labranche et al., 1995; Lodge et al., 1994, 1997).

Materials and methods

Cells

P4.R5 cells, HeLa-based indicator cells engineered to express CD4 and CCR5, were obtained from the AIDS Research and

Fig. 7. Comparison of the C2-V3 regions of NL4-3 and 51-9 Env proteins. The charge difference of NL4-3 with respect to 51-9 is annotated above the NL4-3 sequence using either a minus (−) or plus (+) Periods in the 51-9 sequence indicate identity with NL4-3; curly dashes (∼) denote deletions. The sequence shown is the translation of the region between Stu and Nhel in env. The C2 region of Env begins just upstream of the Stu site. The V3 region is underlined. A small portion of the sequence downstream of V3 is also included.
Variants of pNL4-3 were constructed as follows. The Env clone NL4-3 (pNL4-3) was used for the production of virus. Proviral constructs and L-glutamine. (Accupsin tubes; Sigma). PBMCs were maintained in RPMI separation medium according to the manufacturer’s directions by centrifugation of whole blood through Histopaque-1077 nuclear cells (PBMCs) were isolated from healthy human donors 10% FBS, pen/strep, and L-glutamine. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human donors by centrifugation of whole blood through Histopaque-1077 separation medium according to the manufacturer’s directions (Accupsin tubes; Sigma). PBMCs were maintained in RPMI 1640 (Gibco) medium supplemented with 10% FBS, pen/strep, and L-glutamine.

**Proviral constructs**

A plasmid encoding the complete proviral sequence of HIV-1 clone NL-4-3 (pNL4-3) was used for the production of virus. Variants of pNL4-3 were constructed as follows. The Env tyrosine mutant (NLENVY712A) was made by PCR site-directed mutagenesis of env as previously described (Day et al., 2004). The Env leucine-to-alanine substitution at positions 855 and 856 (OligoNucleotide Water, Zerial (Dresden, Germany). Each image was collected as a Z-series in 0.2 μm steps, deconvolved using a "no-neighbor" algorithm (Slidebook software v.4.0.10, Intelligent Imaging Innovations, Santa Monica, CA.), and processed using Adobe Photoshop.

**Immunofluorescence microscopy**

The following antibodies were used: murine anti-gp41 (Advanced Biotechnologies, Inc., Columbia, MD), murine FITC-conjugated anti-CD63 (BD Pharmingen, San Diego, CA), sheep anti-TGN46 (Serotec, Inc., Raleigh, NC), rabbit anti-EEA1 (Affinity BioReagents, Golden, CO), rhodamine X-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), FITC-conjugated goat anti-rabbit IgG (Jackson), Alexa647-conjugated donkey anti-sheep IgG (Molecular Probes-Invitrogen, Carlsbad, CA). Rab5-GFP, Rab7-GFP, and Rab11-GFP expression constructs were a gift from S. Benichou (Madrid et al., 2005), originally provided by Dr. M. Zerial (Dresden, Germany).

P4.R5 cells were plated (6 × 10⁴) on glass coverslips and were transfected using FuGENE 6 the following day. For each transfection, 1 μg DNA was used. After 24 h, cells were fixed with 3% parafomaldehyde, then permeabiliized with 0.1% NP-40. Cells were incubated with the appropriate normal serum corresponding to the species of the secondary antibody, followed by staining with the appropriate primary, then secondary, antibodies. Glass coverslips were mounted with Gelvatol and imaged with a spinning disc confocal fluorescence microscope (Olympus America, Inc., Melville, NY). Each image was collected as a Z-series in 0.2–0.3 μm steps, deconvolved using a "no-neighbor" algorithm (Slidebook software v.4.0.10, Intelligent Imaging Innovations, Santa Monica, CA.), and processed using Adobe Photoshop.

**Spreading infection assays**

Viral stocks produced as described above from HEK293T cells were normalized by p24 capsid protein concentration. Freshly isolated PBMCs were stimulated with phytohemagglutinin for 3 days. One million cells were pelleted in duplicate and resuspended in 1 ml of RPMI 1640 medium containing 50 ng of virus and interleukin-2 (IL-2) (20 U/ml). After incubating at 37 °C for 6 h, cells were washed and resuspended in 4 ml of medium supplemented with IL-2 (20 U/ml) and then incubated at 37 °C. Samples of medium were taken over time for analysis of viral output by p24 ELISA.
**Viral entry assay**

Viral entry was detected using a flow cytometric assay as described previously (Münk et al., 2002). The assay measures a change in the fluorescence emission from the compound CCF2/AM following cleavage by the enzyme β-lactamase (BlaM). BlaM-containing virions were produced by cotransfection of 293T cells with 10 μg of a proviral plasmid and 10 μg of pMM310, a vector encoding a β-lactamase-Vpr fusion protein (a gift from Nathaniel Landau, originally from Michael Miller, Merck Research Laboratories). Virus-containing supernatants were processed as described above, pelleted at 23,500 × g for 2 h, and resuspended in 1 ml of complete DMEM. After normalization by p24 ELISA, 900 ng of BlaM-Vpr virus was used to infect P4.R5 cells plated at a density of 2.5 × 10^5 cells per well in six-well tissue culture plates. After 5 h, cells were washed with PBS and incubated with 2 μM CCF2/AM (GeneBLAzer Loading kit; Invitrogen)-1% probenecid-25 mM HEPES in 1 ml of serum-free DMEM at 25 °C. After 16 to 18 h, cells were washed, trypsinized, fixed with 1% paraformaldehyde, and analyzed on a MoFlo flow cytometer (Dako Cytomation, Fort Collins, CO) with a UV laser and a 485-nm long-pass dichroic filter with excitation at 351 to 364 nm. Uncleaved substrate was detected as green fluorescence using a 530/40-nm band-pass filter, and cleaved substrate was detected as blue fluorescence using a 450-40 nm band-pass filter.

**Western blot**

Viruses produced as described above from HEK293T cells were used for determination of the incorporation of Env into virions. Filtered viral supernatants were centrifuged at 23,500 × g for 2 h at 4 °C. The viral pellets were resuspended in a small volume of reducing SDS-PAGE sample buffer, and an aliquot was analyzed by ELISA to determine the concentration of p24 antigen. Resuspended pellets were frozen at −20 °C prior to Western blot analysis. Cell lysates of the viral producing cells were harvested by pelleting cells and resuspending them in reducing SDS-PAGE sample buffer. A sample was analyzed for p24 antigen concentration and the lysates were frozen at −20 °C prior to Western blot analysis.

**References**


Day, J.R., Martinez, L.E., Sasik, R., Hitchin, D.L., Dooce, M.E., Richman, D.D., Guatelli, J.C., in press. A computer-based, image-analysis method to bated with a horseradish peroxidase-conjugated secondary antibody, washed, developed with enhanced chemiluminescent substrate (ECL) (Amersham Biosciences, England), and exposed to X-ray film. P24 was detected in the same lanes as either gp120 or gp41 by cutting the membrane and incubating the lower portion separately with the p24 antibody.

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