A tyrosine-based motif in the HIV-1 envelope glycoprotein tail mediates cell-type– and Rab11-FIP1C–dependent incorporation into virions

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Lentiviruses such as HIV-1 encode envelope glycoproteins (Env) with long cytoplasmic tails (CTs) that include motifs mediating interactions with host-cell-trafficking factors. We demonstrated recently that Rab11-family interacting protein 1C (FIP1C) is required for CT-dependent incorporation of Env into HIV-1 particles. Here, we used viruses bearing targeted substitutions within CT to map the FIP1C-dependent incorporation of Env. We identified YW\textsubscript{795} as a critical motif mediating cell-type–dependent Env incorporation. Disruption of YW\textsubscript{795} reproduced the cell-type–dependent particle incorporation of Env that had previously been observed with large truncations of CT. A revertant virus bearing a single amino acid change near the C terminus of CT restored wild-type levels of Env incorporation, Gag-Env colocalization on the plasma membrane, and viral replication. These findings highlight the importance of YW\textsubscript{795} in the cell-type–dependent incorporation of Env and support a model of HIV assembly in which FIP1C/RCP mediates Env trafficking to the particle assembly site.

HIV assembly | HIV envelope | pseudotyping | FIP1C | Rab coupling protein

Lentiviruses such as HIV encode envelope glycoproteins (Envs) with long cytoplasmic tails (CTs) of 150 amino acids or more, whereas avian and murine retroviruses generally encode CTs of 20–30 residues. The reasons for this difference are not entirely clear, but may be attributable to interactions with host-trafficking pathways that define the specificity of Env incorporation into viral particles. A large number of tyrosine- and dileucine-based motifs are present in the HIV-1 Env CT, some of which have been shown to interact with factors involved in vesicular trafficking. The membrane-proximal Yxx\textsubscript{∅} motif (YW\textsubscript{712}) has been well studied and serves as a docking site for the μ-subunit of the clathrin adaptor AP-2 (1, 2). Disruption of this motif enhances cell-surface Env concentration, yet somewhat paradoxically reduces Env incorporation into particles and particle infectivity (3–6). Disruption of YW\textsubscript{802} has also been shown to reduce Env incorporation and infectivity (5, 7). The C-terminal dileucine LL\textsubscript{855} motif interacts with the AP-1 (8) or AP-2 (9) clathrin adaptor proteins and plays a role in endocytosis and in determining the cell-surface levels of Env. We recently performed a systematic mutagenesis of tyrosine- and dileucine-based motifs in the Env CT that confirmed the importance of Y712 on cell-surface levels of Env (3). This study also illustrated an important region of the CT-spanning residues 795–803, in which disruption of YW or LL motifs had dramatic effects on viral replication.

In an important advance to our understanding of the role of the Env CT, Murakami and Freed demonstrated that incorporation of Env into viral particles was cell-type–dependent and that this incorporation in most T-cell lines and macrophages requires an intact long cytoplasmic tail (10). They demonstrated that Env incorporation in 293T cells did not require the long CT, nor was the CT absolutely required for incorporation in particles produced from HeLa or MT-4 cells. However, Env incorporation into particles produced from other T-cell lines and macrophages was severely impaired by CT truncation, and productive replication of virus bearing an Env with a truncated tail was possible only in MT-4 cells. This study strongly implicated host factors in the CT-dependent incorporation of Env.

We recently reported that Rab11-FIP1C (FIP1C) (also known as Rab coupling protein or RCP) and Rab14 are required for Env incorporation and that the effect of FIP1C was dependent upon the Env CT (11). This suggested to us that the cell-type–dependent findings reported by Murakami and Freed (10) may be related to FIP1C-mediated transport of Env mediated through motifs on the Env CT. To test this hypothesis, we examined a panel of viruses bearing mutations of tyrosine- and dileucine-based motifs for their ability to redistribute FIP1C to the plasma membrane. We identified YW\textsubscript{795} as a critical motif that is required for CT-dependent FIP1C redistribution out of the endosomal recycling compartment. Remarkably, the disruption of YW\textsubscript{795} completely recreated the pattern of cell-type dependence on Env incorporation previously observed with CT truncation, and FIP1C depletion had no effect on the level of incorporation of this mutant Env. A downstream second-site revertant was derived that restored Env incorporation and dependence on FIP1C for particle incorporation, suggesting that YW\textsubscript{795} and FIP1C mediate Env incorporation in a cell-type–specific manner.

Results

YW\textsubscript{795} Motif in the gp41 CT Is Required for HIV-1 Env-Mediated Redistribution of FIP1C and for Env Incorporation into Virions. We recently demonstrated that FIP1C is required for HIV-1 Env

Significance

The mechanism of incorporation of the HIV envelope glycoprotein (Env) into a developing particle is not well understood. We used a previously identified cellular trafficking factor, Rab11-FIP1C, as a probe to identify a key motif in the Env cytoplasmic tail that is essential for Env incorporation into particles. We show that this motif governs the cell-type–specific incorporation of Env into particles and the appearance of Env at the particle budding site. Our results provide key insights into how HIV Env is incorporated into budding particles and support an important role for FIP1C in this process.

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Fig. 1. Mapping of HIV-1 gp41 cytoplasmic tail for GFP-FIP1C redistribution and Env incorporation. HeLa cells were transfected with GFP-FIP1C and proviral constructs as indicated in individual panels. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde and stained with human monoclonal antibody 2G12 and fluorescent dye-conjugated secondary antibody. Coverslips were then mounted and images acquired with an overlay motif prevents redistribution of GFP-FIP1C from a predominant perinuclear location in HeLa cells, similar to that previously documented for CT144. This was mutant S5 in Fig. 1A, a YW/SL substitution within a nine-amino-acid stretch (YW 795/SL) of alpha helix 2 of the gp41 CT previously found to be important for Env incorporation and for viral replication in T-cell lines (3, 7). Next, we tested nine individual tyrosine- and dileucine-based motif mutants for incorporation of Env into HIV-1 particles in the H9 T-cell line. Notably, S5 (YW 795/SL) demonstrated a significant defect in Env incorporation (Fig. 1C), whereas no significant defect was observed following disruption of the other eight motifs. We conclude that disruption of the YW795 motif prevents redistribution of GFP-FIP1C and greatly diminishes Env incorporation, suggesting that it may play a role in FIP1C-mediated Env incorporation into particles.

YW/SL595 Virus Replicates Poorly in T-Cell Lines. Murakami and Freed have shown that truncation of the HIV-1 Env CT blocks viral replication in CEM, Jurkat, and MT-2 T-cell lines, as well as in phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cell (PBMCs) and in monocye-derived macrophages (MDDMs) (10). We hypothesized that S5 may recapitulate these results in the absence of tail truncation. We therefore infected H9 T cells with vesicular stomatitis virus glycoprotein (VSV-G)–pseudotyped NL4-3, CT144, S1, S2, or S5 viruses (sequences depicted in Fig. S1 and Fig. 2D) at a multiplicity of infection (MOI) of 0.1 and monitored p24 production in the supernatants over a 3-wk period. As shown in Fig. 2A, S5 and CT144 replicated very poorly in H9 cells, whereas NL4-3, S1, and S2 viruses demonstrated a similar pattern of viral spread, with peak production of p24 around day 18. A very similar pattern of replication was seen for this panel of viruses when introduced into CEM T cells (Fig. S2). This suggested to us that the YW795 motif is required for efficient cell-to-cell spread in nonpermissive cells, similar to that previously documented for CT144.

S5 Revertant Incorporates Wild-Type Levels of Env and Replicates Well in H9 Cells. Despite the apparent lack of replication shown in Fig. 2A, low levels of p24 could be detected in H9 culture supernatants, suggesting that there was ongoing replication at a very reduced level. We continually passed the S5-inoculated H9 T cells until we observed a significant rise in p24 output at 80–83 d postinfection (Fig. 2B). Sequencing of this suspected revertant with VSV-G–pseudotyped viruses as labeled below the blot. HIV particles were harvested, pelleted, and examined for Env incorporation. Arrowhead indicates S5 or YW795/SL mutant that was selected for further evaluation.
and "x-axis timescale. (To further evaluate the significance of the levels of Env restored wild-type Env incorporation in different cell types.

S5 Restores FIP1C-Dependent Env Incorporation and Particle Incorporation. To further evaluate the significance of the levels of Env incorporation exhibited by S5 and SSR viruses in restrictive cells, we measured particle infectivity from supernatants of infected H9 cells. Particles released from CT144- and S5-infected cells were significantly less infectious than wild type, whereas SSR-infected cells released particles that were somewhat more infectious than wild type (Fig. 4A). We previously established that wild-type Env incorporation is dependent upon FIP1C and that FIP1C-dependent Env incorporation requires the intact CT (11). We therefore next asked if the CT revertant mutation in SSR restored FIP1C-dependent Env incorporation. Depletion of FIP1C greatly diminished wild-type Env incorporation in H9 cells as had been previously shown (Fig. 4B). Remarkably, although we could see no effect of FIP1C depletion on the low level of Env incorporation of S5 virus, SSR regained sensitivity to FIP1C depletion (Fig. 4B). In other words, the restoration of cell-type-dependent Env incorporation seen with SSR restored particle Env incorporation to wild-type levels. These results indicated to us that the YW795/SL substitution in the CT completely reproduced the cell-type-specific pattern of Env incorporation seen with a drastic truncation of the CT, whereas the L850S second-site revertant restored wild-type Env incorporation in all cell types tested.

S5 Recreates the Cell-Type-Specific Restriction of CT144 Env Incorporation, Whereas SSR Restores Env Incorporation in All Cell Types. We suspected from the results above that the L850S change in the Env CT had restored efficient Env incorporation in H9 T cells. We next asked whether S5 could completely recapitulate the permissive (293T), semipermissive (HeLa, MT-4), and nonpermissive (H9, CEM, Jurkat, MDM) phenotype with regard to Env incorporation that had been previously established for CT144 (10) and if SSR consistently reversed this restriction of Env incorporation. To test this idea, each cell type was infected with VSV-G-pseudotyped wild-type NL4-3, CT144, S5, or SSR viruses, and Env incorporation into released viral particles was evaluated by Western blot analysis. As shown in Fig. 3A, Env incorporation for each of the four viruses was equal in permissive 293T cells. Both CT144 and S5 Env levels were reduced compared with wild-type NL4-3 in semipermissive HeLa and MT-4 cells, whereas S5 Env restored Env incorporation to wild-type levels (Fig. 3B and C). Remarkably, infection of nonpermissive cell lines and MDMs demonstrated that S5 Env incorporation was restricted similarly to that of CT144 (Fig. 3D–G). In each case, S5 virus revealed conservation of the YW795/SL substitution and a single L850S second-site mutation (depicted in Fig. 2D). Sequencing of the matrix (MA) region of the revertant revealed no changes in this region (wild-type NL4-3 MA sequence). We then reintroduced the L850S in combination with the YW795/SL change into the wild-type NL4-3 background to ensure that this was the relevant change and compared growth of the revertant (termed “SSR”) to that of the S5 virus. As shown in Fig. 2C, SSR replicated well in H9 cells with a growth curve almost identical to that of wild type. This confirmed that the L850S change is a second-site reversion regulating HIV-1 spread in H9 T cells.
SSR Restores Env–Gag Colocalization at the Plasma Membrane. The poor incorporation of S5 Env into virions could be a result of a general disruption of trafficking to the plasma membrane or a disruption of trafficking to specific sites of particle budding. To begin to address this, we infected HeLa cells with VSV-G– pseudotyped viruses at MOI 1.0 and used total internal reflection fluorescence (TIRF) microscopy to analyze Gag and Env distribution on plasma membrane. Both Gag and Env were present in a punctate distribution on the cell surface (Fig. 5). However, the colocalization of Env with Gag puncta was markedly different when comparing wild type with CT144 Env. We noted that Gag particle puncta largely colocalized with Env staining for wild-type virus, whereas colocalization was much diminished with CT144 Env. Using a thresholded Pearson’s coefficient algorithm to quantify colocalization (12), we found a correlation value of 0.52 (±0.09) of Env to Gag for wild-type virus, whereas CT144 colocalization with Gag puncta was reduced to 0.14 (±0.07) (Fig. 5 A and B) (P < 0.01). S5 Env/Gag colocalization was also significantly below that seen for WT at 0.24 (±0.06) (Fig. 5 A and B) (P < 0.01). Notably, S5R Env restored the observed wild-type level of colocalization with Gag puncta (0.53 ±0.06). The roughly threefold difference in colocalization between WT and CT144 or S5 Env with Gag is consistent with the level of Env incorporation seen from immunoblotting (Fig. 3). To further examine the surface distribution of Gag and Env, we performed superresolution microscopy at the level of cell attachment to the coverslip. Results were similar to those seen by TIRF, with marked colocalization of Gag and Env for wild-type and S5R virus and much reduced colocalization for CT144 and S5 (Fig. S.4, rightmost overlay panels).

Cellular levels of Env were next examined on a population basis in infected HeLa cells using flow cytometry. As presented in Fig. S.5, all samples tested expressed high levels of Env protein at cell surface that are well above isotype control-stained cells. WT-, CT144-, S5-, and SSR-infected cells showed similar amounts of Env on the cell surface, with only a very slight shift downward in CT144 and S5 cell-surface levels. This result is generally consistent with a previous study (10), which observed similar levels of WT Env and CT144 Env on plasma membrane in CEM T cells. Thus, the marked differences observed in Env incorporation and in Gag–Env colocalization were not explained by similar reductions in cell-surface Env.

Discussion

The mechanism of specific incorporation of HIV-1 Env into particles remains incompletely understood. Small deletions or substitutions in the globular head of MA reduce or eliminate Env incorporation, a defect that can be rescued when the Env CT is truncated. This suggests that the Env CT and MA interact or, alternatively, that an intact MA and CT are both required for trafficking to specific sites of particle assembly on the plasma membrane. The failure of S5Env to efficiently use the CT and MA to incorporate efficiently into pseudotyped particles is correlated with a dependence on the cellular trafficking factor FIP1C.

We first identified the S5 virus from a panel of mutants in the CT by its loss-of-function phenotype in a GFP–FIP1C redistribution screen (Fig. 1). To complete this analysis using revertant Env, we asked if SSR Env regained the ability to redistribute GFP–FIP1C in HeLa cells. Indeed, although S5 Env was significantly impaired in this redistribution assay, SSR restored a level of FIP1C redistribution equivalent to wild-type Env (Fig. 4D). These data provide additional weight to the idea that FIP1C-dependent trafficking of Env is involved in the cell-type-dependent Env incorporation.
Our findings support the CT-dependent incorporation of Env and highlight the importance of the YW<sub>795</sub> motif on alpha helix 2 of the CT. This motif lies within a predicted alpha-helical segment of the tail that has previously been shown to be important for Env incorporation into particles through deletional mutagenesis (13), and mutations of dileucine- or tyrosine-based motifs in this region severely impair replication in T-cell lines (3). Findings presented here provide important clues to explain the importance of this segment of the CT. The YW<sub>795</sub> mutant failed to redistribute GFP-FIP1C from a predominantly perinuclear location in HeLa cells, suggesting to us that this may be a FIP1C-interacting domain. Rab11-FIP1C is an adaptor protein that dimerizes and forms a heterotetrameric trafficking complex with two copies of Rab11, Rab14, or Rab4 (14–16). We previously showed that FIP1C and Rab14 are required for CT-dependent Env incorporation (11). Although the evidence remains indirect, we propose that a trafficking complex including FIP1C and Rab14 directs Env to the particle budding site through interactions with the Env CT and that YW<sub>795</sub>/SL disrupts this interaction. Following through with this model, a revertant near the end of the CT (L850S) restores FIP1C-dependent trafficking and Env incorporation. One model that could explain our findings is that helix 2 of the Env CT contains a discrete binding site for FIP1C that is disrupted by the YW<sub>795</sub>/SL mutation. A further tenet of this model would be that Env L850S is able to recreate the structural motif required for FIP1C binding and subsequent trafficking to the particle assembly site. We do not have direct binding data to support this model at present, but experiments are in progress to test this possibility. The trafficking model also predicts that we might observe partial colocalization between FIP1C and Env during transit of the complex to the particle budding site. Defining the colocalized populations was not the focus of the current study, and we note that detection of this Env population in static images is complicated by the many compartments in the cell where Env is found, including the endoplasmic reticulum, Golgi, plasma membrane, and endosomes. However, defining the complex of Env and FIP1C using dynamic imaging techniques should be possible and will be the focus of future studies.

A number of models for HIV-1 Env incorporation into particles have previously been proposed, including passive incorporation of Env and Gag, cotrafficking of Env and Gag to a common membrane microdomain, direct interactions between Env and Gag, and indirect interactions through the action of a cellular adaptor molecule (17, 18). Results shown here fit with either the cotrafficking model, in which both Gag and Env traffic independently to a common microdomain on the plasma membrane, or with the indirect interaction model, in which FIP1C or its binding partners could serve as an adaptor directly linking Gag and Env. Our results argue strongly against the passive incorporation model as a relevant model for wild-type Env in most T-cell lines and macrophages, whereas passive incorporation of truncated Env may potentially explain the incorporation of CT144 in 293T or MT-4 cells. We note that the YW<sub>795</sub> motif is highly conserved in clade B strains of HIV-1, whereas at this same position YL is more frequent in clade A and C isolates. A second highly conserved YW motif lies just C-terminal at position 802 and was previously implicated in Env incorporation and in linking Env to TIP47 (19). However, the connection between Env and TIP47 has not been supported in other studies (20).

Results presented here do not yet address the role played by MA in CT-dependent Env incorporation. Tedbury and colleagues recently reported that the HIV-1 matrix mutant Q62R rescues Env incorporation of a wide range of matrix mutants (21). In the trimeric matrix structure, Q62 locates at the trimer interface, and thus changes in this region could modify the overall structure of the MA lattice at a particle budding site and rescue incorporation of the long CT on a purely structural basis.
If steric exclusion of the long CT of Env trimers by mutant MA is the only mechanism at play, however, it is hard to understand how a CT mutant such as YW7580 could lead to exclusion of incorporation into the normal MA lattice in T cells and macrophages, but not in 293T or HeLa cells. We suggest that a cellular trafficking complex including FIP1C plays a specific role in CT-dependent incorporation and that future studies will illuminate the role of components of this complex in linking MA with the Env CT.

Materials and Methods

Cells, Media, and Plasmids. Hela, 293T, and TZM-bl cells were maintained in DMEM containing 10% (vol/vol) FBS and antibiotics. H9, CEM, Jurkat, and MT-4 T-cell lines were cultured in RPMI 1640 supplemented with 10% FBS and 2 mM glutamine and penicillin/streptomycin. MDMs were prepared from human peripheral blood and cultured in media containing GM-CSF as described previously (22). pNL4-3ΔCTdel-144–2 Env was kindly provided by Eric Freed at NCI (Frederick, MD) and referred to here as CT144. A panel of HIV-1 gp41 CT mutants created in the pNL4-3 plasmid backbone has been previously described (3). GFP-FIP1C has been previously described (11). VSVG expression plasmid pHCMV-G was provided by J. Burns at the University of California, San Diego (23). See additional details in SI Materials and Methods.

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