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An amphipathic sequence in the cytoplasmic tail of HIV-1 Env alters cell tropism and modulates viral receptor specificity

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Summary. – The human immunodeficiency virus type 1 (HIV-1) 92UG046 Env protein, obtained from a CD4-independent HIV-1 primary isolate (Zerhouni *et al.*, 2004), has the ability to initiate an infection in HeLa cells expressing CD4 when carrying the full-length (FL) Env, but uses CD8 molecules for receptor-mediated entry when carrying a truncated Env (CT84). To determine whether a specific length or structure in the cytoplasmic tail (CT) is responsible for this alteration of tropism, we compared a series of Env constructs with different CT truncations and the presence or absence of an amphipathic alpha- helical sequence. We found that truncated constructs containing the alpha-helical LLP-2 structure in their CT domains conferred a switch from CD4 to CD8 tropism. The results support the conclusion that the structure of the CT domain can play an important role in determining receptor specificity.

Keywords: HIV-1; Env glycoprotein; gp41; gp120; cytoplasmic tail truncation; alpha-helical LLP2 sequence; CD4, CD8; receptor specificity

Introduction

Prevention and treatment of infection induced by human immunodeficiency virus type 1(HIV-1) is a worldwide public health goal, and a better understanding of HIV-1 tropism is important for development of HIV-1 inhibitors and vaccines. HIV and simian immunodeficiency virus (SIV) Env glycoproteins possess an unusually long cytoplasmic tail (CT) sequence, and acquisition of CT truncations by premature stop codons, which remove almost the entire CT, is a potential mechanism used by HIV or SIV for adaptation to new cell types or escape from effects of entry inhibitors (Kodama *et al.*, 1989; Mulligan *et al.*, 1992; Tilton and Doms, 2010; Waheed *et al.*, 2007). The size and sequence of the gp41 cytoplasmic domain can also modify the biological properties of the Env protein (Vzorov and Compans, 2011). Although SIVmac239 virus with a FL Env is lymphocytotropic, infection of macaques by a molecular clone SIV/17E-Fr, with a truncated cytoplasmic tail of 48 aa, led to disseminated infection (Mankowski *et al.*, 1997). The cytoplasmic tail of the transmembrane protein (TM) also has an essential role in other important viral functions (Berlioz-Torrent *et al.*, 1999; Bowers *et al.*, 2000; Sauter *et al.*, 1996) and contains multiple motifs including three highly conserved alpha-helical "lentivirus lytic peptide" domains (LLP-1, LLP-2, LLP-3) engaged in association with the plasma membrane, reducing bilayer stability, changing membrane ion permeability, and mediating cytopathicity (Chen *et al.*, 2001; Chernomordik *et al.*, 1994; Comardelle *et al.*, 1997; Kalia *et al.*, 2003; Miller *et al.*, 1993; Srinivas *et al.*, 1992; Venable *et al.*, 1989).

CD4-independent variants of HIV-1 were reported with FL and truncated Envs coexisting as quasispecies, which were able to infect $CD4^+$ or $CD8^+$ cells (Zerhouni *et al.*, 2004). A truncated cytoplasmic tail (84 aa) due to a point mutation in TM was observed in an HIV-1 T8-tropic isolate, and patients with elevated levels of such truncated Env variants showed more rapid progression of infection, and greater resistance to antiviral therapy (Waheed *et al.*, 2007; Zerhouni *et al.*, 2004).

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Abbreviations: CT = cytoplasmic tail; FL = full-length; HIV-1 = human immunodeficiency virus type 1; LLP-1,-2,-3 = conserved alpha-helical "lentivirus lytic peptide" domains; SP = signal peptide sequence; SIV = simian immunodeficiency virus; SU(s) = surface subunit(s); TM = transmembrane protein; WT = wild type

In the present study, we used cloned HIV-1 92UG046 Envs and viral pseudotypes to investigate the structural features of HIV-1 92UG046 Env-CT84, with a truncated 84 amino acid cytoplasmic tail, and the biological properties of HIV-1 with this naturally occurring truncated Env, and compared them with HIV-1 92UG046, which possesses the FL CT. We further investigated different forms of HIV-1 92UG046 Env, including mutants with different lengths and structure of CT domains, on their incorporation into pseudotyped virions, syncytia formation activity and receptor/coreceptor mediated entry.

Materials and Methods

Cells. 3T3T4X4, 3T3T4R5, NIH3T3, HeLaT4, HeLaT8 and HeLa cells were obtained from the AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS (NIH). TZM-bl cells (derived by J. Kappes and X.Wu and contributed by Tranzyme, Inc. Durham, NC) (Wei *et al.*, 2002) were obtained through the NIH ARRRP. Hep2, CV-1, 3T3T4X4, 3T3T4R5, NIH3T3, 293T, HeLaT4, HeLaT8, HeLa, and TZM-bl cells were maintained as described (Vzorov *et al.*, 2007). Human blood samples were obtained from volunteers enrolled in the Emory University Institutional Review Board approved "Emory Vaccine Center's Healthy Adults Study" (Protocol #555–2000). Volunteers in this study were healthy adults, aged from 23 to 62, who had signed a written informed consent form. Human PBMCs were separated by centrifugation of whole blood using LSM Lymphocyte Separation Medium (ICN Biomedicals Inc., Costa Mesa, CA). Cells were then stimulated with concanavalin A (Con A, 5 μg/ml in RPMI 1640 containing 10% heat-inactivated fetal calf serum; human interleukin 2 (hIL-2), 10 U/ml; 10 mmol/l HEPES; and antibiotics) for 2 days before infection.

Plasmids and viruses. For molecular cloning, cell-free HIV-1 92UG046 (GenBank Acc. No. AY623600) was obtained from the NIH ARRRP. HIV-1 DNA was extracted from infected human PBMCs using a DNeasy Tissue kit (Qiagen, Valencia, CA) and the *env* gene sequence was amplified by PCR. Two types of 92UG046 Env constructs were compared: wild type (WT) constructs with original *rev* and signal peptide (SP) sequences; and modified constructs with the *rev* and SP replaced by those of NL4-3, which were used to optimize the expression of FL and truncated Envs. The sequence of the *env* gene was identical to that of a CD4-independent HIV-1 clone (92UG046-T8) with a truncated Env protein (GeneBank: AY623600), with several mutations occurring after cell culture passage: K4M, N17D (SP sequence); A461T (gp120); and M620I, K774R (gp41). The FL Env has a mutation in a stop codon at position 784 to a tryptophan (trp) codon. Recombinant vaccinia virus vTF7-3 and the wild type vaccinia virus strain IHD-J were kindly provided by Bernard Moss (NIH, Bethesda, MD) and were propagated and titrated on CV-1 cells. The plasmid pGINT7 β-Gal was provided by Edward Berger (NIH). The plasmid pSG3 delta Env backbone and the pNL4-3.Luc.R^-.E^- delta Env, Vpr backbone with the luciferase gene inserted into the pNL4-3 nef gene were obtained from the NIH ARRRP. The plasmid vector pCAGGS (kindly provided by Y. Kawaoka Madison, WI) containing a CMV/β-actin chimeric promoter or pcDNA3.1(+) (Invitrogen Corporation, Camarillo, CA), were used to express Env constructs. The genes encoding the Rev and FL Env protein of 92UG046 virus were amplified and inserted in the plasmid vector for transient transfection experiments.

Conventional and real-time PCR amplification for HIV. Conventional and real-time PCR amplification were used to determine the entry of pseudotyped virions with FL or truncated Envs. Quantification of proviral DNA from infected cells was performed by real-time PCR using the TaqMan amplification system as described (Kim *et al.*, 2001; Schmidtmayerova *et al.*, 1998; Vzorov *et al.*, 2007). For PCR amplification of the HIV-1 *gag* and 2-LTR U5/U3 regions, primers and probes modified with the fluorescent dye FAM were obtained from Life Technologies (Grand-Island, NY). For the HIV-1 gag region, forward and reverse PCR primers were HIVgagF 5'-AGGACTCGGCTTGCTGAAG-3' and HIV-gagR 5'-GCCTCCGCTAGTCAAAATTTTTGG-3', respectively. The HIVgag probe 5'-CCCCTCGCCTCTTGC-3' was modified with the fluorescent dye FAM. For the HIV 2LTR U5/U3 regions, PCR was carried out using forward and reverse PCR primers 2LTRSG3U5/U3F 5'-TCCCTCATACCCTTTTAGTCAGTGT-3' and 2LTRSG3U5/U3R 5'-CAAGGATATCTTGTCTTCTTT GGGAGAA-3'. The fluorogenic 2LTRSG3U5/U3 probe used was 5'-TTCCATGCTAGAGATTTT-3'. DNA samples corresponding to equal numbers of cells infected by pseudotyped virions were analyzed in parallel. Fluorescence was recorded as a function of PCR amplification cycle. Quantitative HIV determinations were made by comparison with a standard curve produced by using serial dilutions of plasmid DNA (Desire *et al.*, 2001).

Quantitative real-time PCR analysis. The reaction was carried out on a 96-well optical plate (Life Technologies) in a 20 μl reaction volume containing 1/20 of the total DNA extracted from $3x10^5$ cells using TaqMan Universal PCR Mastermix (Roche, Branchburg, NJ). All sequences were amplified using the Applied Biosystems 7500 Real-Time PCR System with the program: 50^0C for 2 min, 95^0C for 10 min, followed by 45 cycles at 95^0C for 15 sec, and 60^0C for 1 min. Samples were tested in duplicate in parallel with the housekeeping gene alpha-tubulin, using primers and probes modified with the fluorescent dye FAM (obtained from Life Technologies). For relative quantitation, delta-delta Ct analysis (Desire *et al.*, 2001) was applied to calculate the fold differences between samples.

Analysis of protein expression. Protein expression was carried out using the recombinant vaccinia virus T7 transient expression system. Briefly, Hep2 cells were seeded in culture dishes and grown to 90% confluence overnight. The cells were then infected with recombinant vaccinia virus VTF7-3 (which expresses the T7 polymerase) at a multiplicity of infection (MOI) of 0.1 for 2 hr followed by transfection with indicated DNA constructs using FuGene 6, obtained from Roche (Indianapolis, IN) At 40 hr post-infection/

transfection, cells were starved in Met, Cys-deficient DMEM for 30 min, and then labeled with [^{35}S]-Met, Cys-labeling mix (obtained from Amersham, Piscataway, NJ) for 3 hr. Surface expression of the HIV Env protein was detected by a surface biotinylation assay as described previously (Vzorov and Compans, 2011). Env expression levels were determined with polyclonal antibodies (HIV-Ig) and Western blotting (WB) or Radioimmunoprecipitation (RIP) methods.

Colorimetric lysate cell fusion assay. Dishes (60-mm diameter) of subconfluent NIH3T3 cells were infected for 1 hr with the vTF7-3 virus at an MOI of 1–2 and then transfected with 4 μg of plasmids for expression of Env constructs. A second population of 3T3T4R5 cells was infected with wt vaccinia virus strain IHD-J and transfected with the pGINT7 ß-Gal plasmid, which contains the ß-galactosidase (ß-Gal) gene under the control of the T7 promoter. At 16 to 20 hr post-transfection, the two cell populations were suspended, mixed in a 96-well tissue culture plate and incubated for 2 hr 30 min at 37^{0}C, after which cell fusion was quantitated by a colorimetric lysate assay (Nussbaum *et al.*, 1994). The data were analyzed with the Delta Soft II Microplate analysis program.

Pseudotype virus generation. Pseudotyped virions were produced by co-transfection of 293T cells with a pSG3 delta Env or pNL4-3. Luc.R$^-$.E$^-$ delta Env , Vpr backbone and Env constructs using calcium phosphate precipitation. After 3 days, supernatants were clarified by centrifugation at 3500 rpm for 15 min and analyzed by titration in TZM-bl cells or RT-PCR in HeLaT4/HeLaT8 cells. Concentrated stocks of pseudotyped virions were prepared by filtration through a 0.45 μm syringe filter and pelleting by centrifugation, and stored at -80^{0}C until use. Prior to infection, pseudotyped virions were treated with 200 U/ml RNase-free DNase I in growth medium containing 10 mmol/l $MgCl_2$ for 30 min 37^{0}C to remove any contaminating proviral DNA.

Analysis of Env protein incorporation into pseudotyped viruses. Samples were analyzed by SDS-PAGE on 8%, 10% and 4–15% acrylamide gels and Western blotting using polyclonal human plasma antibody (HIV-Ig) for analysis of Gag and Env proteins and developed by an ECL kit obtained from Amersham (Piscataway, NJ). The amounts of proteins were quantitated by densitometer analysis (NIH Image version 1.54).

Virus infectivity and neutralization assays. A single-cycle infectivity assay (Kimpton and Emerman, 1992) using Env pseudotyped virus and TZM-bl cells was used to assess the infectivity and neutralization capacity of the FL 92UG046 variant, as previously described (Vzorov and Compans, 2000). Infectivity was also measured 24 hr post-infection by conventional PCR and monitored by quantitative real-time PCR. We used the infectious index (IU/ng), which is the ratio between DNA number copy and core antigen, to compare infectivity titers.

CD4/CD8 receptor blocking assay.At 24 hr before infection, HeLaT4, HeLaT8 or TZM-bl cells (7x10^4 cells/60 mm dish treated and 6x10^4 cells/60 mm dish untreated with 2 μg/ml aphidicolin) (Huberman, 1981) were pre-incubated with 1 μg/ml or 5 μg/ml of mouse anti-CD4 clone RPA-T4, isotype control (IgG1) (Invitrogen Corporation, Camarillo, CA), mouse anti-CD4 clone Q4120 (Sigma-Aldrich, St. Louis, MO) or anti-CD8 clone B9.11 (Beckman Coulter, Brea, CA) for 1 hr at 4^{0}C. Antibody-treated and untreated cells (3x10^5) were inoculated with CT84 or FL 92UG046 Env pseudotyped virions with an infectious index (IU/ng) of 1 or 10, respectively, in medium with 2 μg/ml aphidicolin and 15 μg/ml DEAE-dextran. After 24 hr, DNA samples prepared from HeLaT4 or HeLaT8 cells were used for PCR or RT-PCR analysis. Infectious viral units in TZM-bl cells were determined by counting Tat-dependent β-gal expression-positive infected cells colonies by microscopy.

Competitive binding ELISA assay. ELISA plates were coated with 2 μg/ml of a murine monoclonal antibody against CD4 (OKT4) or CD8 (OKT8) (eBioscience, San Diego, CA) as described before (Vzorov and Compans, 2011). A 100-μl volume (corresponding to 1 μg/ml) of human recombinant CD4 (Protein Sciences Corp., Meriden, CT) or CD8a (22aa–131aa) (Abnova, Walnut, CA) was added per well and incubated for 2 hr. After five washes with PBS containing 0.05% Tween 20, plates were incubated with a mixture of pseudotyped virions and anti-CD4 (clones RPA-T4, Q4120), or anti-CD8 (clone B9.11), or control IgG1 antibodies in assay diluent (ZeptoMetrix corporation, Buffalo, NY). After washes the captured pseudotyped virions were probed with biotin-conjugated polyclonal antibody to HIV-1. The 96-wells plates were developed with tetramethylbenzidine (TMB) and absorbance (OD) determined at 450 nm.

Statistical analysis. Statistical analyses of means with standard deviation (SD) were performed using GraphPad Prism version 5.0 (GraphPad Software Inc.)

Results

Construction of HIV-1 Env mutants with different CT lengths

To examine whether the length of the cytoplasmic tail affects the biological properties of the Env protein, we compared 92UG046 *env* genes with a FL CT, the CT-84 truncation, and a short cytoplasmic tail CT-17 (Fig. 1). We also constructed modified *env* genes, in which the LLP-2 region was fused to the C-terminus of the truncated 92UG046 Env CT17 or to the C-terminus of 92UG046 Env CT62 (with a truncation located prior to LLP-2). In addition four conserved isoleucine/leucine residues of LLP2 were converted to alanine in the constructs designated CT17LLP2-4A or CT62LLP2-4A (Fig. 1).

Effects of modification of CT domains on Env cell surface expression

The synthesis and processing kinetics of the truncated Env proteins were similar to those of FL wild type Env.

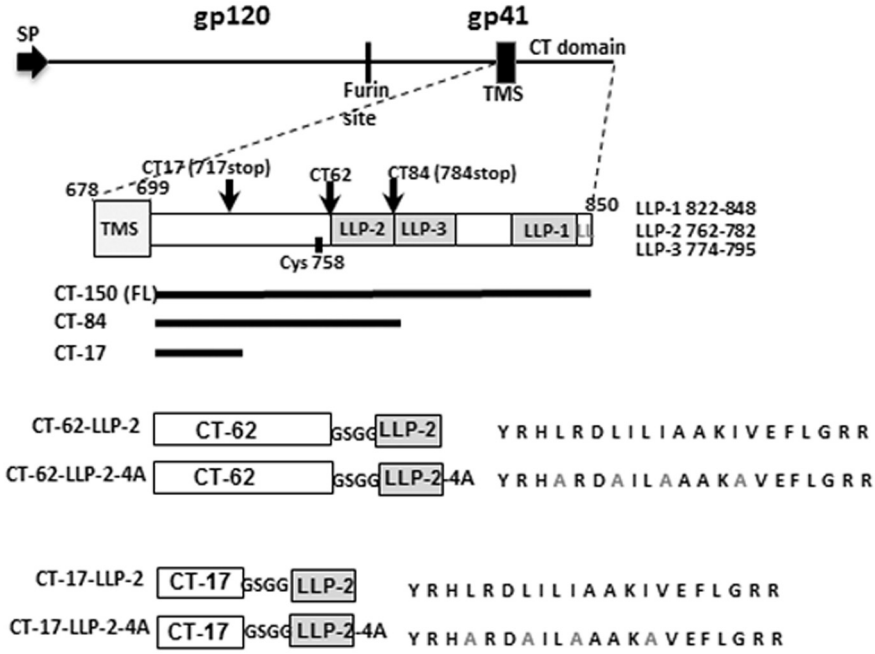

Fig. 1

Schematic diagram of the FL and truncated CT domains of 92UG046 Env

(Top) FL HIV-1 92UG046 envelope glycoprotein containing the signal peptide (SP), the furin cleavage site, the gp120 surface (SU) and gp41 transmembrane (TM) subunits. The CT domain is shown with five membrane-interactimg regions depicted. The upper sequence is LLP-2; the bottom sequence is the LLP2-4A mutant with A residues substituted for 4 conserved L and I residues. CT sequences of Env constructs are indicated as white boxes. Transmembrane-spanning domain (TMS) and LLP sequences are shown as gray boxes. A linker between CT domains (761 Ser/CT84; 716 Pro/CT17) and LLP sequences is GSGG. The positions of stop codons for CT84 and CT17 and the last C-terminal residue of CT62 are shown by arrows. The FL CT domain is a result of cell culture mutation of a stop codon at position 784 to a tryptophan (trp) codon (Zerhouni *et al.*, 2004).

The ratio of SUs to TM subunits expressed on cell surfaces was also similar for all constructs including the wild type 92UG046 Env (not shown). The truncated Env protein CT17 was expressed on the cell surface at levels similar to that of the FL Env as determined by surface biotinylation and quantitation by phosphorimager analysis (Fig. 2a,b). The CT84 Env was expressed at 86% of the level of FL Env. The mutant glycoproteins with 4 A substitutions in their CT domains were expressed on the cell surface at similar levels as those observed with CT62LLP2 Env. The lowest surface expression level was observed with CT17LLP2 Env, which was about 33% of the CT62LLP2 level (Fig. 2c,d). The results show that most truncated Env proteins exhibit efficient transport and cell surface expression. However, the addition of the LLP2 domain to CT17 impaired its transport and cell surface expression.

Syncytia formation activity of FL and truncated Envs

To compare cell-cell fusion activity of the Env proteins and to determine the co-receptor usage, the vaccinia T7 expression system was used to express WT and mutant Env glycoproteins in NIH3T3 cells and determine expression of a β-Gal reporter gene in target cells (3T3T4X4 or 3T3T4R5) with HIV-specific receptors and coreceptors (CD4, CXCR4 or CD4, CCR5). No cell fusion was observed after expression of any of the Env proteins in 3T3T4R5 cells (Fig. 3). The CT-17 truncated Env exhibited about 20% of the fusion activity of FL Env in 3T3T4X4 cells, whereas the CT84 Env did not exhibit any fusion activity using these target cells (Fig. 3). Taken together, the results show differences between the FL, CT84 and CT17 truncated Envs: CT84 Env exhibited a profound reduction of cell-cell fusion activity in $CD4^+$ cells, whereas FL (WT Env) exhibited high and CT17 Env showed low syncytia formation activity. The results also indicate that 92UG046 Envs with FL or truncated CTs exhibit CXCR4 coreceptor usage.

Effects of CT domains on Env incorporation into pseudotyped virions

To evaluate the effects on Env incorporation into pseudotyped virions, we compared Env constructs with FL and truncated CT domains. We transfected 293T cells and three days post-transfection, supernatants from equal amounts of cells were analyzed. The replacement of WT with NL4-3 SP

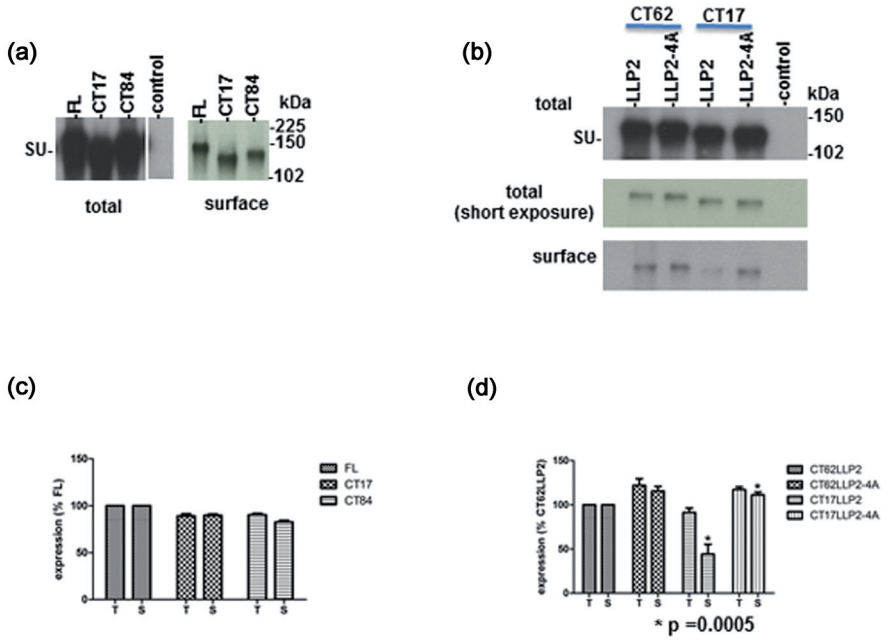

Fig. 2

Surface (S) and total (T) cell expression of FL and truncated Env proteins (92UG046) in Hep2 cells

Hep2 cells were infected by vaccinia vTF7-3 virus and then transfected by T7 plasmid constructs. At the end of the labeling period, the cell surface proteins were biotinylated, lysed and analyzed as described in Methods. All 92UG046 Env constructs (a) or modified 92UG046 Env constructs (c) possess the rev and env signal peptide of NL4-3 HIV-1. The levels of total or surface proteins (SU of Env) present in the autoradiograph relative to the FL 92UG046 (b) or relative to CT62LLP2 (d) were quantitated by phosphorimager analysis as described in Methods. Data in c and d are plotted as the means of three experiments. Error bars represent standard deviations. Numbered lines in a and b (right) indicate locations of molecular weight standards.

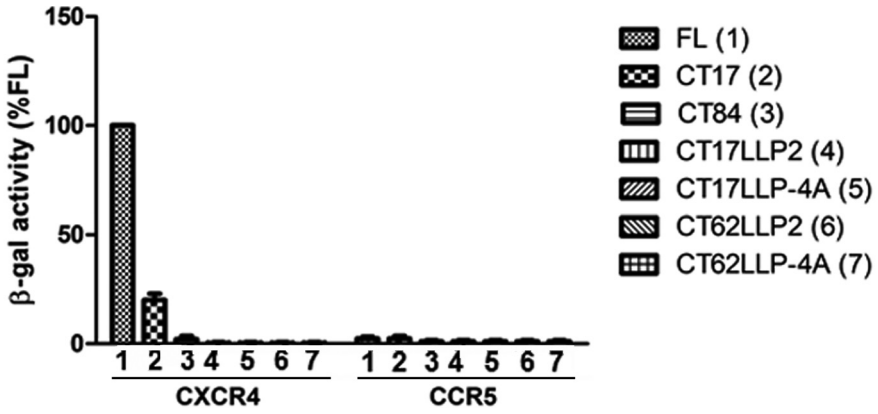

Fig. 3

Cell fusion activity of Env 92UG046 with FL or truncated CT sequences

NIH3T3 cells were infected with a vaccinia vTF7-3 virus and then transfected by T7 plasmid constructs. 3T3T4R5 or 3T3T4X4 cells were infected with IHD-J vaccinia virus and transfected with a reporter gene construct. At 18 hr post-transfection, the two populations were mixed and incubated, after which cell fusion was quantitated by a colorimetric lysate assay. The data shown are the percentages of the ß-Gal activity observed in WT (FL Env)-expressing cells**.** Data are plotted as the mean of three experiments. Error bars represent standard deviations.

did not affect levels of Env incorporation. All truncated Env proteins with the NL4-3 SP were found to be incorporated into pseudotyped virions at levels about 6 to 11% of the wild type FL Env (Fig. 4a,b). The mutant Env proteins (CT17LLP2, CT17LLP2-4A, CT62LLP2, or CT62LLP2-4A) were found to be incorporated into pseudotyped virions at about the same levels as the parental CT17 or CT84 Env, but at a significantly lower level than FL Env. The intensity of the p24 protein

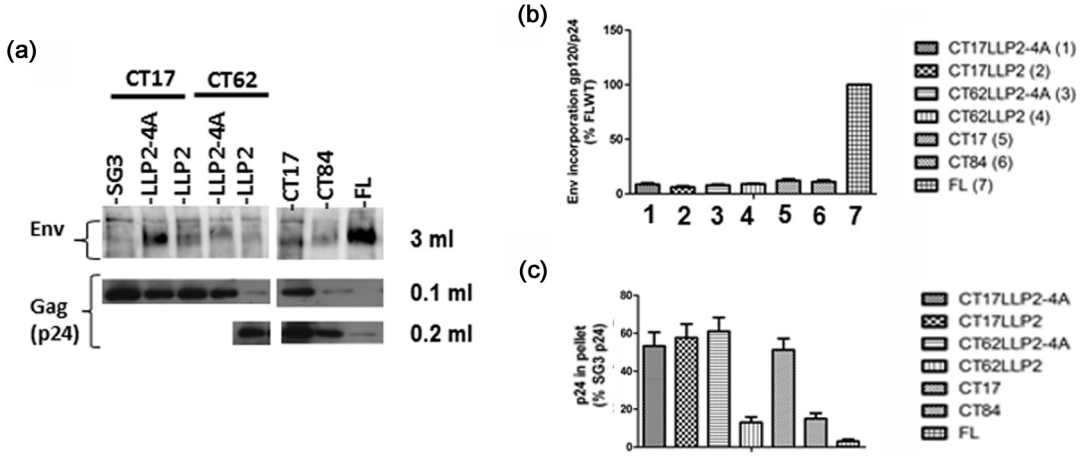

Fig. 4

Effects of CT domains on production and Env incorporation into pseudotyped virions

(a) Virions pseudotyped with 92UG046 Env proteins were obtained by co-transfection of 293T cells with Env plasmid constructs and with a plasmid expressing the *env*-minus molecular clone SG3 (HIV-1) using a ratio of Env plasmids to SG3 of 3 : 1 and collected and purified after 3 days. The same amount of medium (0.1 ml, 0.2 ml, 3 ml) from equal amounts of cells was used in all samples except for SG3, which used 1/5 of the amount of medium of other samples. The samples were analyzed by 8% SDS-PAGE and Western blotting by using a polyclonal antibody from human plasma (HIV-Ig) for analysis of Gag and Env proteins. The amounts of proteins were quantitated by densitometer analysis (NIH Image version 1.54) for each mutant (b, c). The Env/Gag ratio was determined and indicated as % of the FL Env level (100%) (b). The Gag level was determined and indicated as % of the SG3 Gag level (100%) (c). Data are plotted as the mean of three experiments. Error bars represent standard deviations.

bands in CT17LLP2, CT17LLP2-4A, CT62LLP2-4A pseudotyped virions was about a 30-fold higher than the p24 level in virus pseudotyped with FL Env (Fig. 4c). In contrast, the p24 levels of virions pseudotyped with CT84 or CT62LLP2 were about 5-fold higher than with FL Env (Fig. 4c). Taken together, the results indicate that FL and truncated Env were

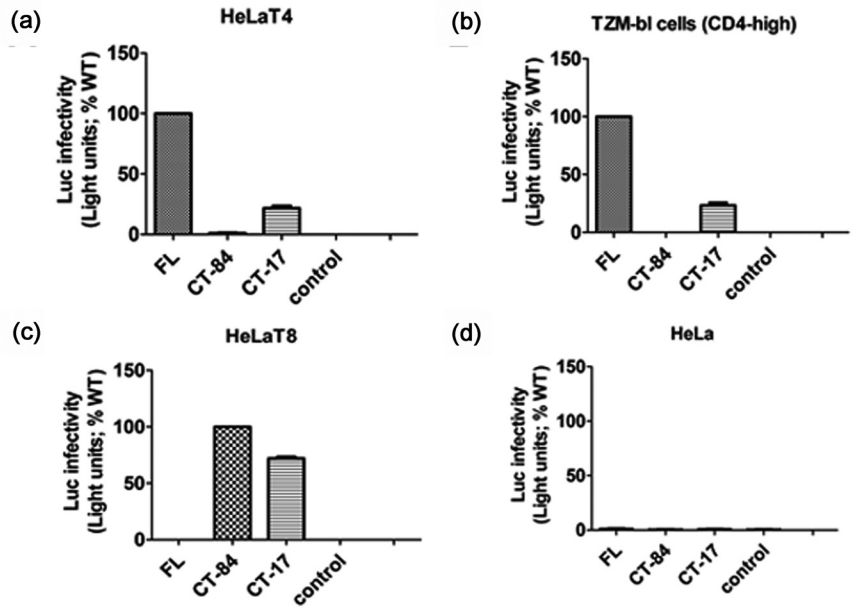

Fig. 5

Entry of virions pseudotyped with HIV-1 92UG046 Env with FL or truncated CT into TZM-bl, HeLaT4, and HeLaT8 cells

Single-round infections were performed in HeLa or derivative HeLa cells with CD4 or CD8 receptors using a replication-defective NL4-3 virus bearing the luciferase reporter pseudotyped with FL CT84, CT17 envelopes. Luciferase activity was calculated after comparing luciferase activity from extracts of cells infected with FL (100%) (a, b) or with CT84 (100%) (c). Uninfected cells were used as control. The results are shown with the standard deviation (n = 3).

incorporated into pseudotyped virions at different levels and the LLP sequence selectively affected this process.

Effects of CT length on entry of virions pseudotyped with 92UG046 Env

To compare the entry of virions pseudotyped with FL or truncated Env 92UG046 into cells with different receptors, we compared HeLaT4 cells (expressing low CD4 levels), TZM-bl cells (expressing high CD4 levels) and HeLaT8 cells (expressing CD8). HIV infectivity was determined by β-galactosidase activity of a modified β-galactosidase gene driven by the HIV-1 long terminal repeat (LTR) promoter and transactivation by the viral Tat protein in TZM-bl cells (Kimpton and Emerman, 1992) or by infection with luciferase-encoding HIV-1 pseudotyped virions (He *et al.*, 1995). Virions pseudotyped with CT17 92UG046 Envs were able to enter cells with CD4 or CD8 receptors (HeLaT4, HeLaT8 and TZM-bl cells) (Fig. 5). Virions pseudotyped with FL 92UG046 Env were able to enter cells with CD4 receptors (HeLaT4 or TZM-bl cells) and infected $CD4^+$-HeLa cells with high efficiency. In contrast, virions pseudotyped with CT84 Env were only able to infect HeLaT8 cells by using CD8-mediated entry. However, the efficiency of infection of the viruses pseudotyped with CT84 Env was relatively low, about 100 relative luciferase units per $1x10^4$ cells. We also observed that replacement of the SP sequence by that of NL4-3 reduced the infectivity of Env FL pseudotyped virions about 1000-fold, but no such effect was observed with CT84 Env pseudotyped virions. The results indicate that the CT domain has a significant effect on productive entry of 92UG046 HIV-1 Env pseudotyped virions into HeLa cells with specific receptors, and that the length of the CT domain can confer an effect on infectivity.

Effects of antibodies on entry of FL or truncated Env pseudotyped virions in CD4-high, CD4-low, or CD8 HeLa cells

We hypothesized that the CT84 truncation of the cytoplasmic domain confers a conformational change to the external domain of Env, which is responsible for the observed differences in tropism of the 92UG046 Env proteins. To further investigate the effect of the CT84 truncation on receptor specificity of the Env protein, we used a blocking assay for entry into HeLaT4 or HeLaT8 cells by anti-CD4 (clones Q4120, RPA-T4) or anti-CD8 monoclonal antibodies. Non-dividing cells were arrested in the G_1-S phase of the cell cycle by using 2 μg/ml of aphidicolin (Huberman, 1981). In HeLaT8 cells treated with anti-CD8 antibodies, infection by virions pseudotyped with CT84 Env was reduced compared to untreated HeLaT8 cells (Fig. 6a,b). However, we observed that anti-CD8 antibody had no effect on infection using virions pseudotyped with FL Env. Anti-CD4 (Q4120, RPA-T4) antibodies did not block entry of pseudotyped virions with modified or unmodified Envs in HeLaT4 or HeLaT8 cells. In contrast to RPA-T4, Q4120 Ab was able to block infection by virions pseudotyped with FL Env in TZM-bl cells (Fig. 6d). We also observed that anti-CD8 partially blocked the entry of CT62LLP2 pseudotyped virions into HeLaT8 cells but had no effect on the entry of virions pseudotyped with CT62LLP2-4A (Fig. 6c), which may reflect disruption of the helical sequence of the LLP2 domain of CT62LLP2 Env. The anti-CD4 and anti-CD8 antibodies were also ineffective at blocking infection by virions pseudotyped with Env CT17. These results further indicate that the structure and length of the CT domain can modify the receptor specificity for infection by HIV-1 Env.

Effect of antibodies on binding of FL or truncated Env pseudotyped virions to CD4 or CD8 receptors

We compared effects of antibody on binding of FL and truncated Env pseudotyped virions to CD4 or CD8 proteins to understand the mechanism by which the CT domain modulates the efficiency of infection of cells. The binding of pseudotyped virions was measured by competitive binding ELISA in the presence of anti-CD4 or anti-CD8 monoclonal antibodies or a control antibody. The CT84 and CT17 pseudotyped virions efficiently recognized CD4 or CD8 molecules, whereas binding of FL Env pseudotyped virions was blocked by anti-CD4 antibodies (Fig. 7a,b). The binding of truncated CT17, CT84 and FL Env pseudotyped virions to CD4 was reduced in the presence of anti-CD4 (Q4120) antibodies (about 35% to 75% reduction) but no such effect was seen with the RPA-T4 antibody (Fig. 7c,d,e). Notably, binding of CT84 Env pseudotyped virions to CD8 was reduced in the presence of anti-CD8 antibodies (about 74% reduction).

Discussion

Previous studies have shown that truncation of the CT can have various biological effects on the HIV Env protein, including increasing its expression levels on the cell surface, enhancing incorporation into VLPs, and promoting cell fusion activity (Johnston *et al.*, 1993; Ritter *et al.*, 1993; Spies and Compans, 1994; Vzorov and Compans, 1996; Zingler and Littman, 1993). The main goal of the present study was to investigate the possible effects of changes in the cytoplasmic domain on the alteration of HIV cell tropism. Our hypothesis that the cytoplasmic tail plays an important role in determining HIV-1 tropism is based in part on previous observations: (i) truncation of the CT domain alters the conformation of the external domain (Spies *et al.*, 1994; Vzorov and Compans,

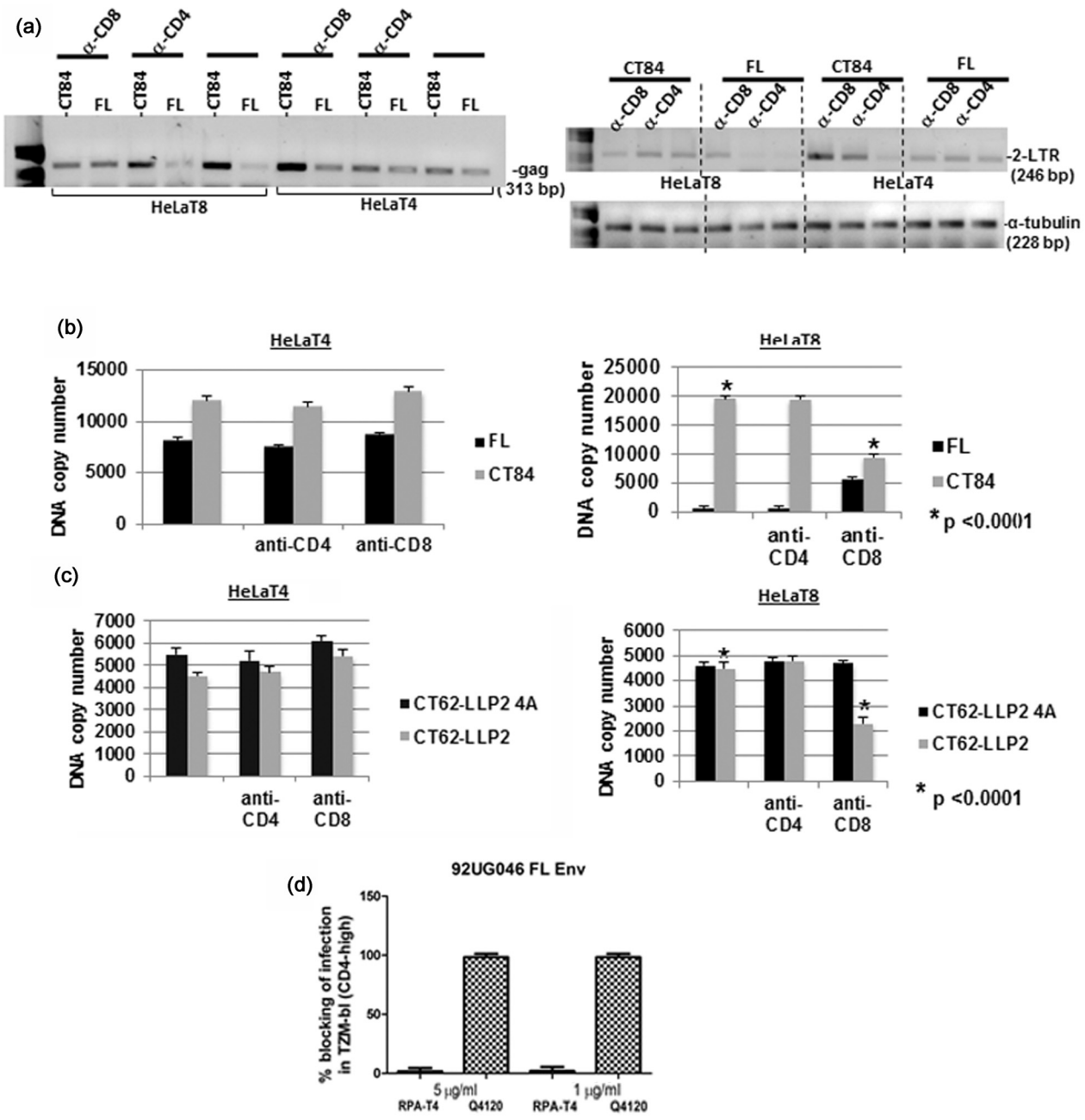

Fig. 6

Blocking of infection by 92UG046 Env pseudotyped virions using mAbs RPA-T4, Q4120, or B9.11

Aphidicolin- treated HeLaT4 or HeLaT8 (a, b, c) or untreated TZM-bl (d) cells were incubated with antibodies for 1 h at 4^0C and infected with CT84 or FL 92UG046 Env pseudotyped virions with 1 or 10 infectious index (IU/ng p24 Gag), respectively (a, b, c), or 30 infectious particles of pseudotyped virions per well (d). (a, left panel) viral entry was determined by PCR amplification for the HIV SG3 RGag region 24 hr post-infection. (a, right panel) Viral entry determined by PCR amplification for the HIV 2-LTR circle 24 hr post-infection; PCR analysis of the α-tubulin gene in cell lysates was used to standardize DNA recovery. (b, c) Viral entry determined by RT-PCR amplification for the HIV SG3 RGag region 24 hr post-infection. (d) Viral infectivity determined by a single-cycle (TZM-bl) infectivity assay. Data are plotted as the mean of three experiments and error bars represent standard deviations (b, c, d). Asterisks denote significant differences of the HIV-1 DNA copy numbers in HeLaT8 cells treated with anti-CD8 Abs compared to untreated HeLaT8 cells infected by CT84 or CT162LLP2 ($p < 0.0001$ by unpaired Student's *t*-test).

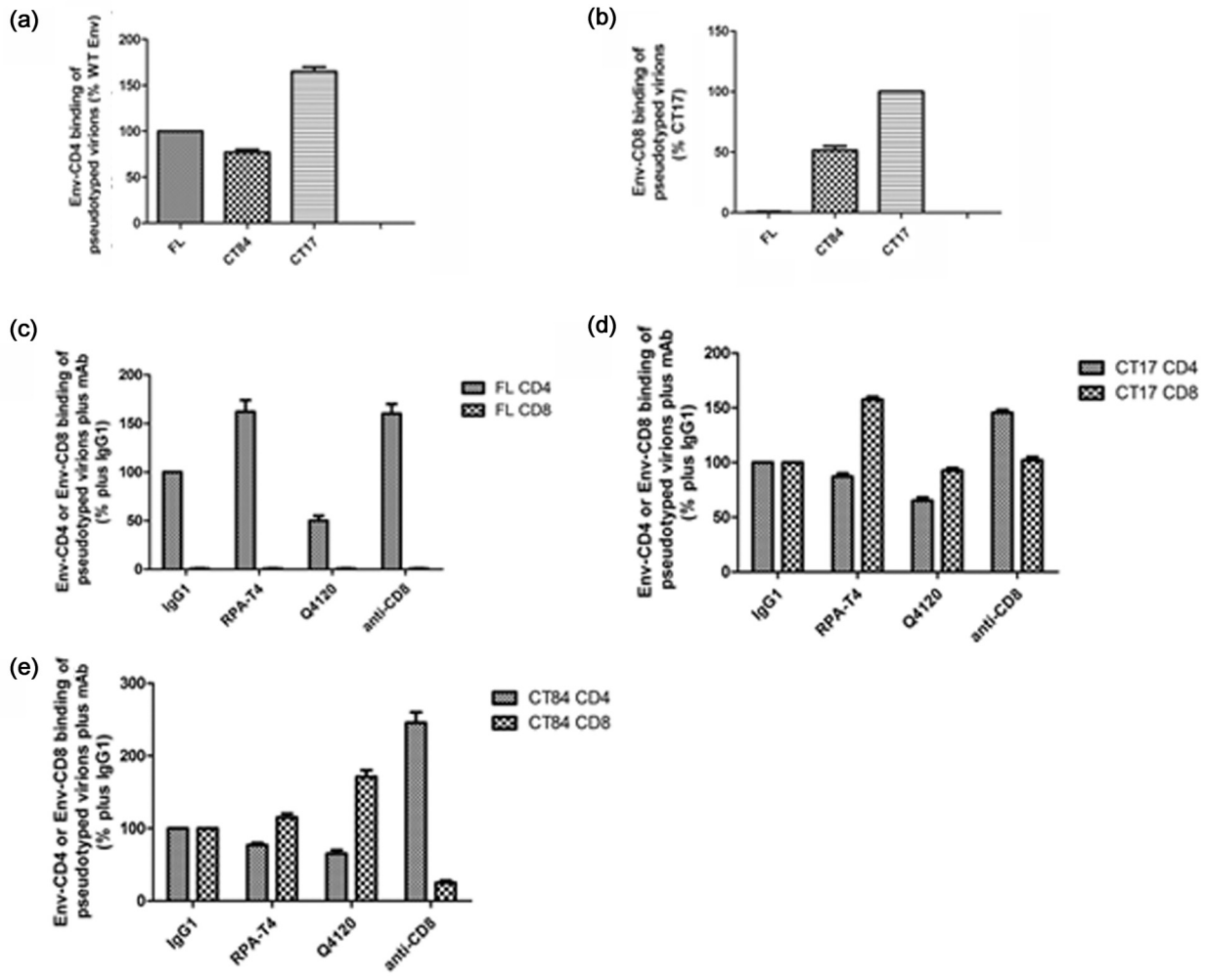

Fig. 7

Effects of antibodies on binding of FL or truncated Env pseudotyped virions to CD4 or CD8

A 96-well plate was coated with OKT4 (a, c, d, e) or OKT8 (b, c, d, e) antibody and with soluble CD4 or CD8, and incubated with virions pseudotyped with Env in the presence of anti-CD4 or CD8 antibodies. After extensive washes the bound virions were detected by biotin-conjugated polyclonal antibody to HIV-1. Results represent percentage of bound virions in the presence of anti-CD4 or anti-CD8 Ab compared to that observed for bound virions in the presence of mouse IgG1 (100%). The results are shown with the standard deviation (n = 3).

2000); (ii) changes in phenotypic characteristics of the SU are affected by CT length (Vzorov *et al.*, 2005); (iii) the length and sequence of the CT domain can impact viral biological properties including trimer stability, syncytia formation activity and infectivity (Bonavia *et al.*, 2005; Edwards *et al.*, 2001; Taylor *et al.*, 2008; Vzorov and Compans, 2011; Wyss *et al.*, 2005). In this study we show that the tropism of FL 92UG046 Env changes from a CD4-high phenotype to a CD8-tropic phenotype when a truncated form of Env is used to initiate an infection. The precise sequence, which were important to change the 92UG046 Env tropism were not determined in previous studies (Saha *et al.*, 2005; Zerhouni *et al.*, 2004). Moreover, the authors reported that CD4-independent virus isolates (including HIV-1 92UG046-T8), which they obtained after infection of purified $CD8^+$ T cells, were not inhibited by anti-CD8 antibodies, in contrast to previously reported $CD8^+$ T cell tropic viruses (Saha *et al.*, 2001). In one study CD4-independent variants were isolated from 7 of 12 patients through infection of purified $CD8^+$ T cells, and one CD4-independent variant isolated from a patient (92UG046-T8) had a sharply truncated cytoplasmic tail (CT84) due to a point mutation (Zerhouni *et al.*, 2004). To investigate the sequences responsible for differences in tropism, we compared two types of CT truncations: an exten-

sive truncation, which deleted membrane-interactive regions such as amphipathic helical regions LLP-1, LLP-2, and LLP-3 (Chen *et al.*, 2001; Chernomordik *et al.*, 1994; Comardelle *et al.*, 1997; Kalia *et al.*, 2003; Miller *et al.*, 1993; Srinivas *et al.*, 1992; Venable *et al.*, 1989) and shorter truncations located within specific amphipathic helical regions (60 to~150 aa). Many isolates of HIV-2 and SIV contain stop codons that truncate the CT to within 15 to 20 aa of the membrane-spanning domain, which have been shown to occur upon passage of the virus in culture (Hirsch *et al.*, 1989; Kodama *et al.*, 1989; Mulligan *et al.*, 1992). Macaques inoculated by SIV1A11 with a cytoplasmic tail of 18 aa demonstrated transient viremia (Luciw *et al.*, 1992), while infection of macaques by an SIV17E variant with a longer cytoplasmic tail of 48 aa led to disseminated infection (Mankowski *et al.*, 1997). In HIV-1, some variation of size in the Env CT has also been observed. HIV-1 (KB-1) carrying an 18aa Env CT was isolated from a Japanese male hemophiliac (Shimizu *et al.*, 1992). HIV-1 isolates that show CD4 independence bear extensive truncations in their gp41 CTs, located prior to the membrane-interactive regions (Edwards *et al.*, 2001, 2002). SIV with a truncated Env-CT17 exhibits a defect in early steps of replication in non-dividing cells, and demonstrates an extended range of cells susceptible to infection (Vzorov *et al.*, 2007).

$CD4^+$ or $CD8^+$ HeLa cell clones provide a sensitive system for quantitatively comparing infectivity of M-tropic and T-cell-tropic isolates of HIV-1, including primary patient isolates (Platt *et al.*, 1998). HIV binds at higher levels to HeLa cells than to other cells. A low gp120-CD4 interaction is masked by a more avid gp120-heparan sulfate interaction in HeLaT4 cells (Mondor *et al.*, 1998). Viral entry studies have also been conducted using HeLa cell lines expressing different levels of CD4 and CCR5 viral receptors (Platt *et al.*, 1998). Reduced CD4 levels were a significant barrier for entry of cells by HIV-1 clones with certain V1/V2 modifications (Walter *et al.*, 2005).

We observed differences in entry levels of FL, CT84, CT17, or modified Env pseudotyped virions in HeLaT4, HeLaT8 or TZM-bl cells. We found that the addition of the LLP-2 sequence to the truncated CT62 construct resulted in the ability to infect HeLaT8 cells, while the corresponding virions pseudotyped with this construct containing 2 L and 2 I to 4 A mutations were unable to infect these cells, indicating that the helical structure of LLP2 sequence is important for this process. Moreover, the LLP sequence and length of the CT domain may affect selective incorporation of Env glycoproteins into viral particles. Most importantly, we observed that LLP sequences in the CT domain may change the receptor specificity of the Env protein. These results support the conclusion that the LLP-2 sequence and the structure of the cytoplasmic tail play important roles in determining the receptor-specificity of the 92UG046 HIV-1 Env protein. Anti-CD8 antibodies were only able to inhibit entry of CT84 pseudotyped virions in HeLaT8 cells, suggesting that the CT84 Env determines the receptor specificity for infection of these cells. Anti-CD8 antibodies did not inhibit HIV-1 92UG046-T8 entry in $CD8^+$ cells in a previous study (Zerhouni *et al.*, 2004) but a blocking effect was demonstrated in the present study. This difference may be due to using virions pseudotyped with a cloned CT84 Env in our experiments. The incomplete blockage observed with anti-CD8 antibodies may also be due to ability of HIV-1 virions to bypass receptor requirements and enter through endocytic pathways (Fredericksen *et al.*, 2002; Wei *et al.*, 2005).

In conclusion, we believe that the present results provide the first evidence that a specific sequence in the CT domain can modulate viral receptor specificity of HIV-1 Env. We hypothesize that the CT84 truncation in the CT domain confers CD8-tropism by inducing conformational changes in functional regions of gp120. Such changes may enhance exposure of receptor-binding segments in gp120, resulting in viruses that differ in their cell tropism and biological properties.

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