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Cytoplasmic domain effects on exposure of co-receptor-binding sites of HIV-1 Env

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Abstract

We defined the effects of the cytoplasmic domain (CT) of the Env glycoprotein on co-receptor usage of HIV-1 by reciprocal exchanges of regions containing V3–V5 loops between CD4-dependent and CD4-independent isolates. Primary HIV-1 isolate Env clones CD8 CXCR4-tropic 92UG046 CT84 with an 84-aa truncated CT domain, CD4 CXCR4-tropic 92UG046, and CD4 CCR5-tropic SF162 with full-length (FL) CT domains were used for comparison. The parental 92UG046 Env with CT84 was not fusogenic, but a chimeric SF162 V3–V5-CT84 with an 84-aa truncated CT domain, which demonstrated a switched co-receptor specificity, exhibited syncytium-formation activity with 3T3T4X4 cells. The wild-type (WT) SF162 Env with CT84 or full-length CT was fusogenic in 3T3T4R5 cells. By exchange of V3–V5 loops, we were able to alter WT SF162 to switch its co-receptor preference, which was not dependent on CT domain length. These results provide evidence that CT domains can induce conformational changes in functional regions of gp120 and determine receptor tropism but do not modulate HIV-1 coreceptor specificity.

Introduction

Specific regulation of membrane fusion is important in biological functions such as viral entry. Specific viral surface glycoproteins are responsible for promoting membrane fusion, binding of the virus to cell-surface receptors, and sometimes to co-receptors. The HIV Env glycoprotein is a complex of two subunits: the surface gp120, which mediates binding of the virus to specific cell-surface receptors, and the gp41-surface-transmembrane-cytoplasmic subunit, which promotes membrane fusion [1, 5, 7].

The native, pre-fusogenic form of Env is thought to be present in a metastable state [20, 32–34]. A conformational change of the Env protein is triggered either by receptor binding or by inside-out signaling from the CT domain [10, 13, 17, 28, 33]. CT modifications can affect

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Compliance with ethical standards

Conflict of interest The author(s) declare that they have no competing interests.

the conformation of the external domain of gp41 on the cell surface [24] and may enhance exposure of receptor-binding segments in gp120 [29].

Recent results indicate the involvement of other gp120 regions (such as V1, V2, and C4) and even the gp41 external domain [6, 12] in co-receptor binding. The present study was designed to investigate possible effects of the CT domain on modulation of co-receptor usage.

Materials and methods

Cells, plasmids, viruses

TZM-bl, 3T3T4X4, 3T3T4R5 and NIH3T3 cells were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS (NIH). Hep2, CV-1, 3T3T4X4, 3T3T4R5, NIH3T3, 293T, and TZM-bl cells were maintained as described [27]. The recombinant vaccinia virus vTF7-3 and the wild-type (WT) vaccinia virus strain IHD-J were kindly provided by Bernard Moss (NIH, Bethesda, MD) and were propagated and titrated on CV-1 cells. Plasmid pGINT7 β -Gal was provided by Edward Berger (NIH, Bethesda, MD). Plasmid pBlueSF162 containing the SF162 env gene (accession number EU123924) was provided by Sang-Moo Kang (Georgia State University, Atlanta GA). Plasmids containing the 92UG046 env genes (accession number AY623600) with different CT domain sequences were described before [29]. Plasmid vector pCAGGS (kindly provided by Dr. Y. Kawaoka) containing a CMV/ β -actin chimera promoter was used to express Env constructs. Plasmid containing the HIV backbone pSG3 delta env was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS (NIH).

Analysis of protein expression

Protein expression was carried out using the recombinant vaccinia virus T7 transient expression system [19]. 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 h followed by transfection with the indicated DNA constructs using FuGene 6, obtained from Roche (Indianapolis, IN, USA). At 40 h post-infection/transfection, cells were starved in Met, Cys-deficient DMEM for 30 min, and then labeled with [³⁵S]-Met, Cys-labeling mix obtained from Amersham (Piscataway, NJ, USA) for 3 h. Surface expression of the HIV Env protein was detected by a surface biotinylation assay as described previously [28]. Briefly, after labeling and chase, cells were washed with PBS at 4 °C and then incubated with 1 ml of NHS-SS-Biotin dissolved in PBS (1 mg/ml) for 30 min at 4 °C. After labeling and surface biotinylation, the cells were lysed with lysis buffer and proteins then precipitated overnight at 4 °C with a polyclonal human plasma antibody (HIV-Ig) plus protein A agarose beads obtained from Pierce (Rockford, IL, USA). Proteins bound to protein A agarose beads were washed three times with lysis buffer, solubilized with 10 % SDS and heated at 95 °C for 5 min. Dissociated proteins were precipitated again with streptavidin agarose beads at 4 °C for 3 h and then washed three times with lysis buffer. Protein samples were then prepared by addition of reducing sample buffer and heated at 95 °C for 5 min prior to analysis by SDS-PAGE. The gels were fixed, dried, and exposed to phosphor screens for quantification of the

surface and total glycoproteins ratios using a phosphorimager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software.

Cell fusion assays and colorimetric lysate assay

Dishes (60-mm diameter) of subconfluent NIH3T3 cells were infected for 1 h 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 β-Gal plasmid, which contains the β-galactosidase (β-Gal) gene under the control of the T7 promoter. At 16 to 20 h post-transfection, the two cell populations were suspended, mixed in a 96-well tissue culture plate and incubated for 2 h 30 min at 37 °C, after which cell fusion was quantitated by a colorimetric lysate assay [19]. The data were analyzed with the Delta Soft II Microplate analysis program.

Pseudotype virus generation and analysis of Env protein incorporation

Pseudotyped virions were produced by co-transfection of 293T cells with the HIV backbone pSG3 delta env proviral clone, which contains a defective vpu gene, and Env CMV/β-actin promoter plasmid 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 purified as described [29]. Briefly, concentrated stocks of pseudotyped virions were prepared by filtration through a 0.45-µm syringe filter and pelleting by centrifugation and stored at –80 °C until use. The purified samples were analyzed by SDS/PAGE on 8 % or 10 % acrylamide gels and Western blotting by using a polyclonal antibody from human plasma (HIV-Ig) for analysis of Gag and Env proteins and developed using an ECL kit obtained from Amersham (Piscataway, NJ, USA). The amounts of proteins were quantitated by densitometry (NIH Image version 1.54).

Virus infectivity assay

A single-cycle infectivity assay [14] using Env-pseudotyped virus and TZM-bl cells was used to assess virus infectivity. To compare infectivity of virus stocks by titration, two methods were compared: using similar amounts of p24 for input and using amounts of virus that give a similar infectious index (IU/ng), which is the ratio between the number of copies of proviral DNA and core antigen, thereby taking both of these characteristics into consideration [27, 29].

Statistical analysis

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

Results

Construction of chimeric HIV-1 Envs with different CT lengths

To further investigate the role of specific CT sequences in cell tropism, we generated chimeric Envs by reciprocal exchanges of regions containing V3–V5 loops between CD8

CXCR4-tropic HIV-1 92UG046 CT84 with an 84-aa truncated CT domain, CD4 CXCR4-tropic HIV-1 92UG046, and CD4 CCR5-tropic SF162 with full-length (FL) CT domains (Fig. 1).

V3 loop phenotypes of chimeric HIV-1 Envs

Several specific amino acids (which correlated with CD4 independence, R5 or X4 determinants) were observed in the V3 loop sequences of the two isolates, 92UG046 and SF162 (Fig. 1, Table 1). The N6 residue was found in most CD4-independent isolates and was changed to N6E in 92UG046 [35]. Most R5X4 isolates have N6, as found in SF162 Env. The P13 residue correlated with CD4 independence. 92UG046 contains P13, whereas in SF162 it is changed to T, P13T. The motif IGXI (residues 27–30) is essential for interaction with the R5 co-receptor. Most CD4-independent Envs contain the IGXI sequence. In 92UG046, there is a change to KGYI; SF162 also has this motif. R5 tropism is correlated with S11 and D25. SF162 has these amino acids, whereas 92UG046 has R11 and L25. The R11 is an X4 tropic determinant [15]. These data indicate that chimeric 92UG046 and SF162 Envs have different V3 phenotypes.

Chimeric Env proteins with FL or CT84 sequences exhibit high levels of cell-surface expression

The synthesis and processing kinetics of the modified Env proteins were similar to those of the wild-type Env. The ratio of SU to TM subunits expressed on the cell surface was also similar for all constructs, including the wild-type 92UG046 and SF162 Envs (not shown). The Env proteins with CT17 and CT84 truncations were expressed on the cell surface at high levels similar to that of the full-length Env, as determined by surface biotinylation (Fig. 2A, B, and C). The results showed that chimeric FL and CT84 Env proteins exhibit efficient transport and cell-surface expression.

The V3–V5 regions affect syncytium-formation activity of chimeric Envs

To compare cell-cell fusion activity of the Env proteins and to determine their co-receptor usage, we used the vaccinia T7 expression system to express wild-type and chimeric Env glycoproteins (FL, CT17, CT84) in NIH3T3 cells and expression of a β -Gal reporter gene in target cells (3T3T4X4 or 3T3T4R5) with HIV-specific receptors and co-receptors (CD4, CXCR4 or CD4, CCR5). No fusion was observed upon expression of 92UG046 Envs in 3T3T4R5 cells (Fig. 3D). The 92UG046 CT17-truncated Env exhibited about 20 % of the fusion activity of full-length Env in 3T3T4X4 cells (Fig. 3C). The 92UG046 CT84 Env did not exhibit fusion activity using 3T3T4R5 or 3T3T4X4 target cells (Fig. 3C and D). However, 92UG046 Env with full-length or truncated CTs exhibited CXCR4 co-receptor usage (Fig. 3C). The full-length and truncated (CT17, CT84) Envs of SF162 only exhibited cell-cell fusion activity with 3T3T4R5 cells. The SF162 CT17 and CT84 Envs exhibited about 267 % and 98 %, respectively, of the fusion activity of the full-length Env (Fig. 3A). However, the SF162 V3V5-CT84 exhibited about 60 % of the cell-cell fusion activity of full-length Env in 3T3T4X4 cells (Fig. 3B). Other modified Envs (SF162 V3V5-FL, 92UG046 V3V5-FL and 92UG046V3V5-CT84) did not show cell-cell fusion activity. The results indicate that V3–V5 loop sequences of gp120 subunits determine the co-receptor specificity of wild-type or chimeric Env proteins with full-length or truncated CT domains.

CT84 truncation can induce conformational changes in receptor-binding segments in gp120 [13] but it does not affect the co-receptor binding site. The chimeric 92UG046 Envs were observed to be non-fusogenic with CD4CCR5 cells, which may be due to a property of the V1-V2 loops of CD4-independent 92UG046 [35] and a change in the net amino acid charge from +8 to +5 in the CCR5-phenotype V3 loop (Fig. 1).

Effects of gp120 and CT domains on Env incorporation into pseudotyped virions

To evaluate the effects of SU and CT domains on Env incorporation, we compared Env pseudotyped virions with full-length, truncated CT (CT17, CT84) Envs with WT or chimeric SU domains (Fig. 4). WT and chimeric Env (SF162 and 92UG046) with full-length CT domains were incorporated into virions at high levels. Incorporation of CT17 Envs with SU subunits of SF162 or 92UG046 Envs was impaired. The truncated SF162 Env protein (CT84) was found to be incorporated into pseudotyped virions at levels about 110 % of that of wild-type full-length Env. Truncated WT 92UG046 and chimeric CT84 Envs were incorporated at intermediate levels, about 15 % of the level of wild-type full-length Env incorporation. Taken together, the results indicate that the efficiency of incorporation of Env into virions is dependent on both the SU and CT domains.

Effects of CT domains on infectivity of chimeric Env pseudotyped virions

The main goal of this experiment was to compare the effect of the CT domain on the conformational change of gp120 in the receptor-binding site by determination of the infectivity of HIV-1 in cells expressing CD4⁺ CCR5⁺ CXCR4⁺ molecules. Our previous results indicate that the CT84 domain determines CD8-receptor specificity and that the full-length CT domain determines the CD4-receptor specificity of 92UG046 Env [29]. In contrast, CT17 truncation generates defective Env proteins [2, 27, 29]. To compare the infectivity of virions pseudotyped with chimeric Env containing FL or truncated CT domains, we used TZM-bl cells, which stably express large amounts of CD4 and CCR5 and constitutively express CXCR4. These indicator TZM-bl cells do not require late events such as virion protein expression, virus assembly, or virion maturation [14]. The infectivity was compared by titration of pseudotyped virions evaluated by two methods, using similar p24 levels or similar infectious indexes (IU/ng) (described in Materials and methods) [29]. Virions pseudotyped with FL (SF162Env FL, SF162Env V3V5-FL and 92UG046 FL) or truncated CT17 Envs were able to infect TZM-bl CD4 X4R5 cells (Fig. 5). In contrast, virions pseudotyped with CT84 Envs (SF162Env CT84, SF162Env V3V5-CT84, 92UG046 CT84 and 92UG046 V3V5-CT84) were not able to use CD4-mediated entry (Fig. 5). Taken together, the results indicate that the CT domain can modulate receptor specificity, but not co-receptor usage of HIV-1 Env.

Discussion

In this study, we evaluated the role of the CT domain in modulation of co-receptor usage of HIV-1 Env. Recently, we found that 92UG046 Env was CD4-tropic when carrying a full-length Env and changed to CD8 tropism when a truncated Env is used to initiate an infection [29]. The structural features by which the CT domain could alter viral tropism have been defined in previous studies [24, 26, 28]. The role of the cytoplasmic domain in stabilization

of the Env protein and the effects of introducing specific sequences that are predicted to affect trimer stability into the CT domain of the SIV/HIV Env protein have been investigated [28, 30]. The amphipathic LLP2 region or a GCN4 sequence (three-helix bundle) inserted in the cytoplasmic tail significantly affected the function of CD4- and CCR5-binding sites of Env proteins [28, 29].

The third variable region, V3, of gp120 is approximately 35 residues long and has a major influence on HIV-1 tropism [11]. A switch from R5 to X4 usage is driven by V3 mutation and has been linked to an increase in the net positive charge of V3, allowing an interaction with the negatively charged surface of the CXCR4 coreceptor [9, 21, 22]. Dual-tropic HIV-1 with V3 sequences have been identified that were identical to those of R5-tropic variants, indicating that determinants of CXCR4 tropism were present outside of the V3 region [12]. To further investigate the role of specific CT sequences in cell tropism, we compared chimeric Envs with reciprocal exchanges of the region containing the V3 loop from CD4-independent CXCR4-tropic 92UG046 HIV-1 with a truncated CT and that of CD4-dependent CXCR4-tropic 92UG046 or CCR5-tropic SF162 HIV-1 with full-length CT domains. Even though the parental 92UG046 Env with CT84 was not fusogenic, the chimeric SF162 V3V5-CT84, which demonstrated a switch in co-receptor specificity, exhibited syncytium-formation activity with 3T3T4X4 cells. In chimeric Envs that possessed an identical V3 loop, SF162 V3V5-CT84, but not SF162 V3V5-FL, induced formation of syncytia. Co-receptor binding is important for cell-cell fusion activity, as confirmed by assays of fusion activity after reciprocal exchanges of V3 between SF162 and 92UG046 Envs. The co-receptor binding site is not well exposed on virions, even on those with CD4 independent Env proteins [15, 25]. To compare our results by using attachment inhibitors is difficult, because CD4-independent viruses and some HIV-1 envelopes (92UG046) can be resistant to entry inhibitors [16]. However, the present results clearly indicate that alteration of cytoplasmic tail length (FL, CT84, CT17) did not modulate co-receptor usage of WT or chimeric SF162 R5 or 92UG046 X4 Envs. Conformational changes of coreceptor-binding segments in gp120 can be triggered by mutations in the external parts of Env (gp120 or the external domain of gp41) [12].

The transmembrane domain and fusion peptide of Env proteins are required for cell-cell fusion [4, 18]. The Env proteins of SF162 carrying full-length or truncated CT sequences (CT17, CT84) with or without coiled-coil domains in the cytoplasmic tail are capable of forming syncytia. CD4-independent CT17 Env, which lacks coiled-coil domains in the cytoplasmic tail, exhibited syncytium-formation activity in a cell-type-dependent manner [23, 28], indicating that membrane fusion does not appear to be mediated by CT α -helical bundles [3]. However, fusion between the viral and cellular membrane progresses through a series of a transient conformational changes (receptor and co-receptor binding steps) in Env proteins [8], which may be modulated by inside-out signaling from the cytoplasmic tail [31]. The data presented here can be applied for developing Env immunogens for HIV vaccines, in which the cytoplasmic domain of Env proteins can be modified to enhance immunogenicity [30]. Such changes may significantly affect the function of receptor-binding segments in gp120 and enhance exposure of co-receptor-binding sites in modified Env proteins [28].

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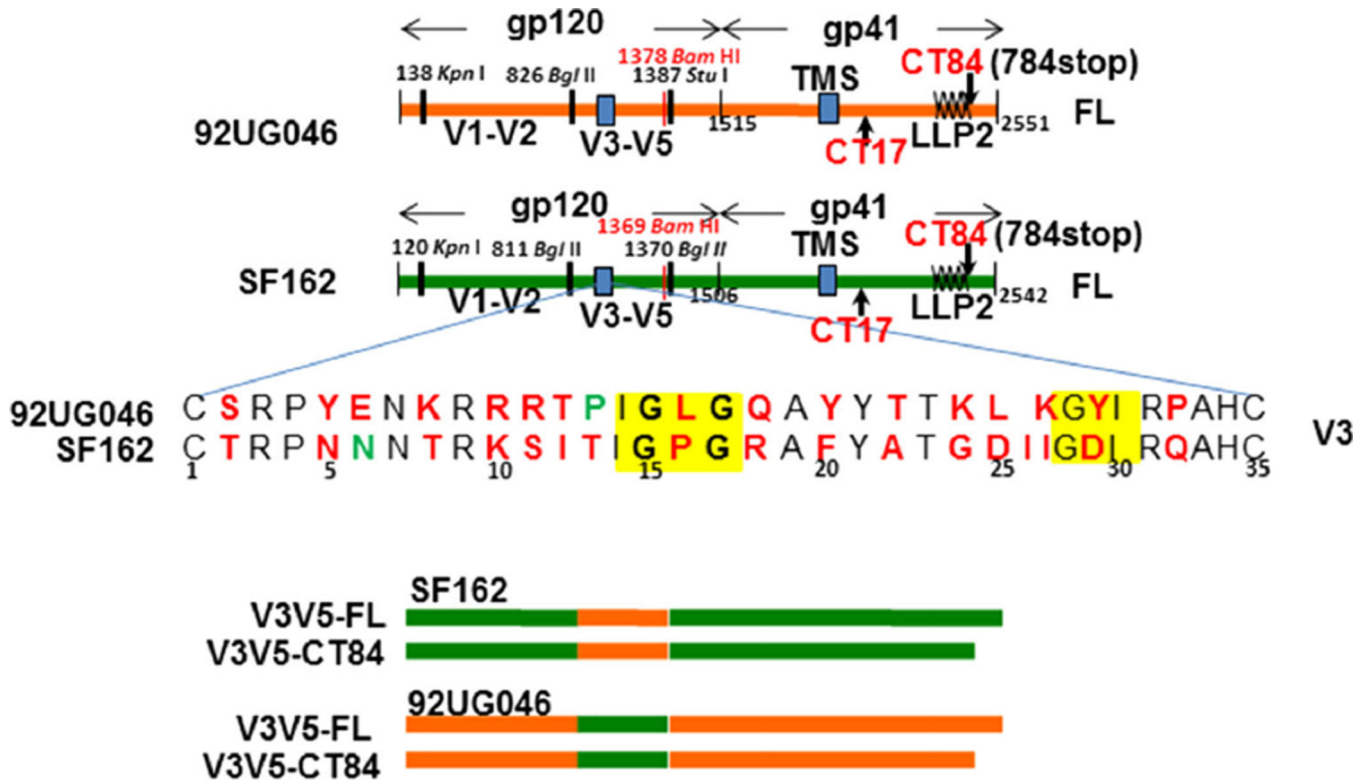
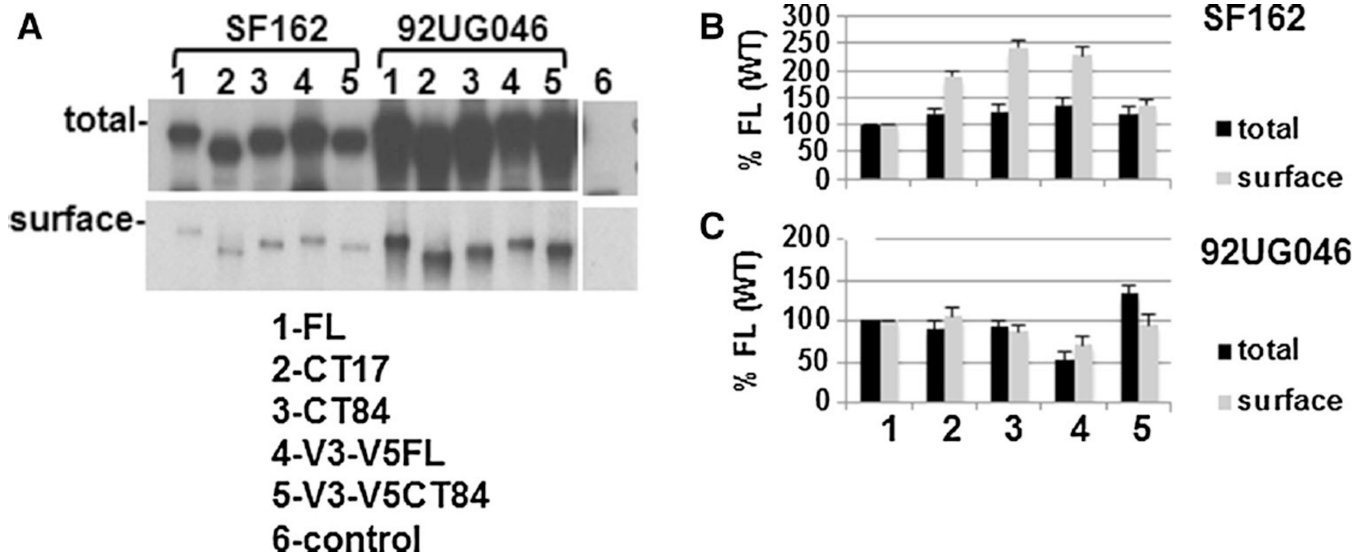


Fig. 1. Schematic diagram of chimeric Envs. (Top) 92UG046 and SF162 FL-Envs. The gp120 and gp41 sequences are depicted. The V3 loop and the membrane-spanning region are indicated by boxes. The stop codons (after LLP2 region) in CT84 (CD8 tropic) or CT17 (before functional motifs) are shown by arrows and in red color. The orange bars indicate 92UG046 Env, and green bars indicate SF162 Env sequences. Also indicated are the locations of *Kpn*I, *Bgl*II, *Stu*I cleavage sites. New *Bam*HI restriction sites for construction of chimeric Envs are shown in red color. The differences between the residues of SF162 and 92UG046 in the V3 sequences are indicated in red. Motifs described in the text are shown by yellow boxes. The residues associated with CD4 independence are shown in green (color figure online)

**Fig. 2.**

Analysis of surface expression of chimeric Env proteins (92UG046 and SF162) in Hep2 cells. (A, B, C) Hep2 cells were infected with vaccinia vTF7-3 virus at an moi of 0.1 and then transfected with T7 plasmid constructs. After 40 h, infected cells were labeled with [³⁵S] cysteine/methionine for 3 h. At the end of the labeling period, the cell-surface proteins were biotinylated and lysed, immunoprecipitated, and precipitated with streptavidin-agarose beads (surface) or only lysed (total) as described in Materials and methods. HIV-specific proteins were immunoprecipitated with a polyclonal antibody from human plasma (HIV-Ig). The T7 plasmid construct without an env gene was used as a control. The levels of total or surface proteins (SU subunit of Env) present in the autoradiograph relative to the FL Env were quantitated by phosphorimager analysis. Data in B and C are plotted as the mean of three experiments. Error bars represent standard deviations

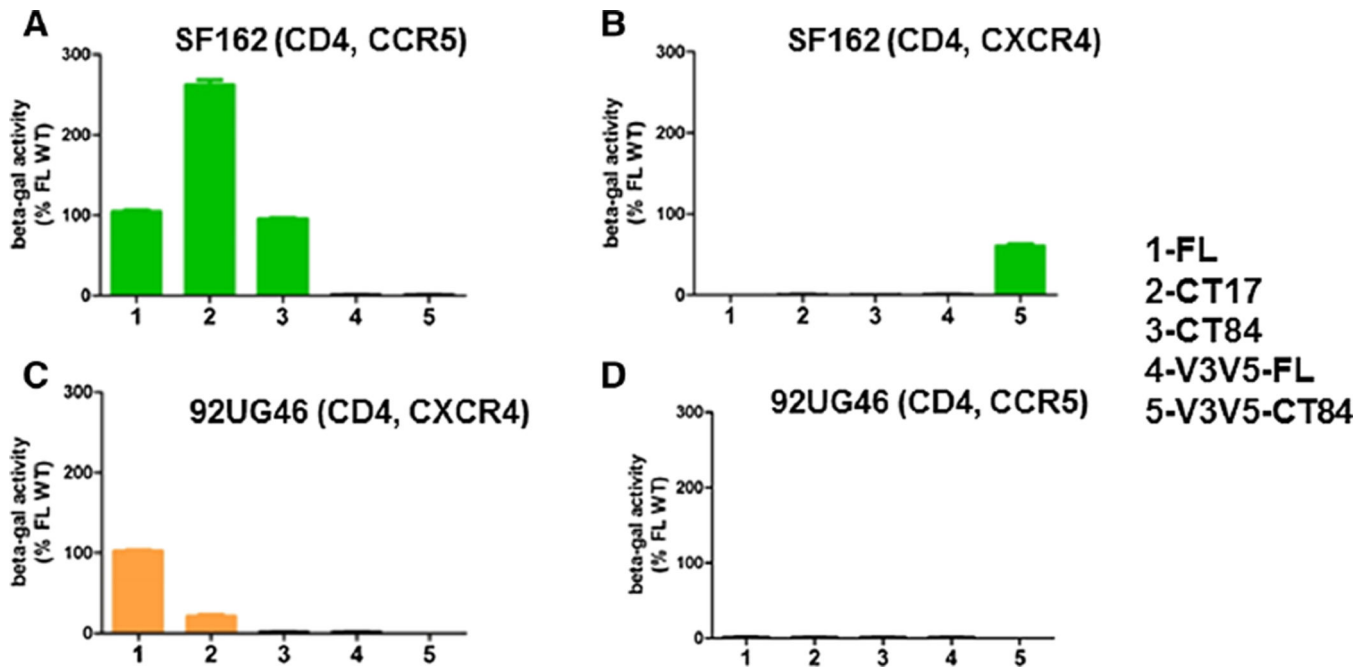


Fig. 3. Effect of SU or CT domains of Env (92UG046 and SF162) on cell-cell fusion activity. A. NIH3T3 cells were infected with a vaccinia vTF7-3 virus and then transfected with T7 plasmid constructs. 3T3T4R5 (A, D) or 3T3T4X4 (B, C) cells were infected with IHD-J vaccinia virus and transfected with a reporter gene construct. At 18 h post-transfection, the two populations were mixed in a 96-well tissue culture plate and incubated for 2 h 30 min at 37 °C, after which cell fusion was quantitated using a colorimetric lysate assay. The data shown are the percentages of the β -Gal activity observed in WT (full-length Env)-expressing cells. A representative is shown from three independent experiments. Error bars represent standard deviations. The green bars indicate SF162Env, and orange bars indicate 92UG046Env cell-cell fusion activity (color figure online)

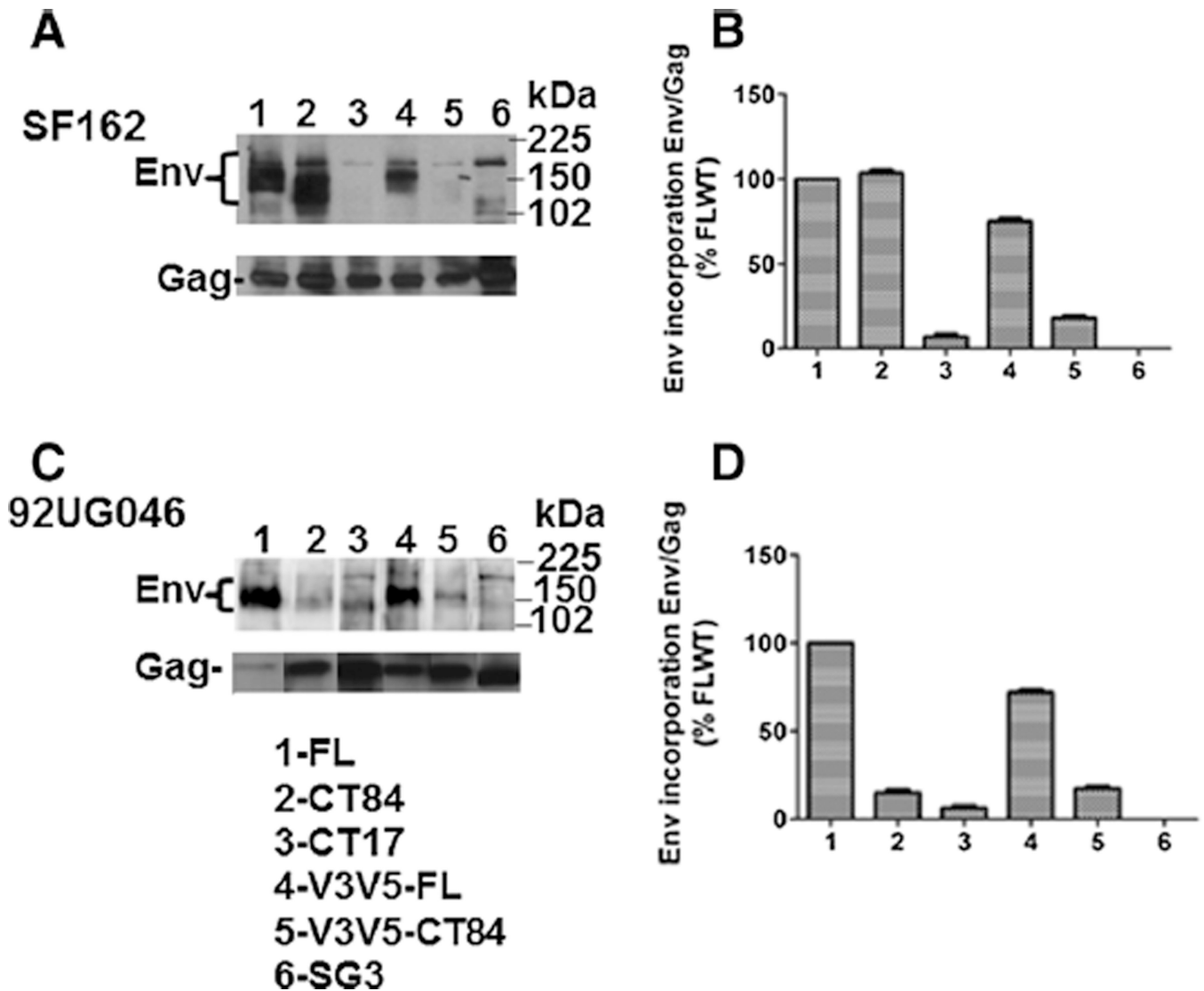


Fig. 4. Effects of gp120 (SU) and CT domains on Env incorporation into pseudotyped virions. Virions pseudotyped with SF162Env (A, B) or 92UG046Env (C, D) proteins were obtained by co-transfection of 293T cells with Env plasmid constructs and with pSG3 plasmid expressing the env-minus molecular clone SG3 (HIV-1) at a ratio of Env plasmids to SG3 of 1 to 1 (A) or 3 to 1 (C) and collected and purified after 3 days. 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. Proteins were quantitated by densitometry (NIH Image version 1.54). The Env/Gag ratio was determined and expressed as a percentage of wild-type Env (100 %) (B, D). Data are plotted as the mean of three experiments. Error bars represent standard deviations

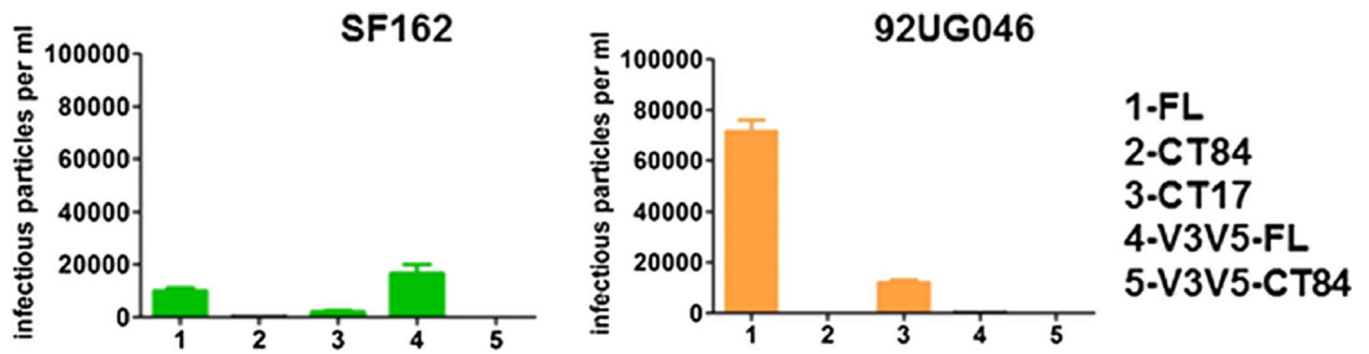


Fig. 5. Effect of CT domains on infectivity of chimeric Env pseudotyped virions. The infectivity of pseudotyped virions was determined by β -galactosidase assays in TZM-bl cells. A representative is shown from three independent experiments. Error bars represent standard deviations. The green bars indicate SF162Env, and orange bars indicate 92UG046Env infectivity (color figure online)

Table 1

V3 phenotypes of 92UG046 or SF162 Envs

92UG046 residue	SF162 residue	Phenotype	Phenotype residue
E6	N6	CD4-independent	N6
E6	N6	R5X4	N6
P13	T13	CD4-independent	P13
KGXI	IGXI	CD4-independent	IGXI
R11	S11	R5-tropic	S11
L25	D25	R5-tropic	D25
R11	S11	X4-tropic	R11

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