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Conserved Structures Exposed in HIV-1 Envelope Glycoproteins Stabilized by Flexible Linkers as Potent Entry Inhibitors and Potential Immunogens†

Yen-Hung Chow,‡ Olivia L. Wei,‡,§ Sanjay Phogat,‡ Igor A. Sidorov,‡ Timothy R. Fouts,|| Christopher C. Broder,§ and Dimiter S. Dimitrov*,‡

Laboratory of Experimental and Computational Biology, National Cancer Institute-Frederick, NIH, Miller Drive, Frederick, Maryland 21702-1201, Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, Maryland 20814-4799, and Institute of Human Virology, University of Maryland Biotechnology Institute, University of Maryland, Baltimore, Maryland 21201

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ABSTRACT: The HIV-1 envelope glycoprotein (Env) undergoes conformational changes while driving entry. We hypothesized that some of the intermediate Env conformations could be represented in tethered constructs where gp120 and the ectodomain of gp41 are joined by flexible linkers. Tethered Envs with long linkers (gp140-14 with 15 aa and gp140-24 with 26 aa) were stable and recognized by conformationally dependent anti-gp120 and anti-gp41 monoclonal antibodies (mAbs). Surprisingly, these proteins potently inhibited membrane fusion mediated by R5, X4, and R5X4 Envs with 5−100-fold lower IC_{50} than a tethered Env with short linker (gp140-4 with 4 aa), gp120, gp140, soluble CD4, or DP178 (T20). Compared to gp140, gp140-14,24 exhibited increased binding to anti-gp41 cluster II mAbs but not to cluster I mAbs. Cluster II mAbs but not cluster I, IV, or V mAbs reversed the inhibitory effect of gp140-14,24 suggesting a role of exposed conserved gp41 structures for the mechanism of inhibition. These findings suggest the existence of conserved gp41 structures that are important for HIV-1 entry and can be stably exposed in the native environment of the Env even in the absence of receptor-mediated activation. Thus, tethered Envs with long linkers may not only be important as HIV-1 inhibitors but also for elucidation of viral entry mechanisms and development of novel vaccine immunogens.

Binding of the HIV-1 envelope glycoprotein (Env, gp120−gp41) complex with CD4 to coreceptor molecules initiates a series of conformational changes that are the heart of the fusion machinery driving viral entry (*1*−*3*). The elucidation of the nature of the Env conformational changes is critical for understanding the mechanism of HIV-1 entry, and also may provide new tools for development of inhibitors and vaccines (*4*−*6*). Complexes of gp120 with CD4 have been used as immunogens and elicited neutralizing antibodies against conformational epitopes induced by CD4 (*7*−*10*). After the discovery of the HIV-1 coreceptors, it has been proposed that coreceptors can induce intermediate Env conformations critical for viral fusion that may include structures conserved among various HIV-1 isolates and be used as vaccines (*11*, *12*). Unlike the CD4 induced conformational changes, which mostly affect gp120, the coreceptor binding to gp120 not only affects gp120 but also would presumably trigger exposure of gp41 intermediates that ultimately cause membrane fusion. Other approaches for enhanced exposure of hidden structures have been also proposed as removal of carbohydrates (*13*, *14*), stable exposure of the coreceptor binding site on gp120 in CD4-independent Envs (*15*), deletion of gp120 variable loops (*16*−*18*), and constructs containing gp41 sequences (*19*−*21*). It appears that a vaccine based on the gp41 molecule has the drawback that neutralizing epitopes of gp41 are rare and/or unfavorably presented to the immune system (*21*). Another major problem in development of vaccines based on the HIV-1 Env is the instability of the gp120−gp41 complex which tends to dissociate to gp41 molecules that likely represent postfusion conformations (*21*). Initial attempts to stabilize the Env complex involved the mutation of the gp120−gp41 cleavage recognition sequence (*22*−*24*). Usually in such proteins, gp41 is truncated to its ectodomain so they are secreted as soluble uncleaved Envs ($gp140_{UNC}$). These proteins are stable and can induce cross-reactive antibodies (*25*−*27*). However, they may not resemble the native intermediate Env structures involved in the HIV-1 entry, and antibodies elicited by their use as immunogens are not very efficient in neutralization of primary isolates. Recently, a different approach was developed where a disulfide bond was introduced between gp120 and gp41 to stabilize the association between these two subunits (*28*). This protein has antigenic properties that resemble those of the virion-associated Env, and its immunogenicity is being evaluated. Two other groups of investigators have recently developed proteins based on N-terminal gp41 helices that show HIV-1 inhibitory activity and have potential as immunogens (*29*, *30*).

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* To whom correspondence should be addressed. Dimiter S. Dimitrov, Laboratory of Experimental and Computational Biology, NCI at Frederick, NIH, Bldg 469, Rm 246, P.O. Box B, Miller Drive, Frederick, MD 21702-1201. Phone: 301-846-1352, Fax:3 01-846-6189. E-mail: dimitrov@ncifcrf.gov.

‡ NIH.

§ Uniformed Services University of the Health Sciences.

|| University of Maryland.

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Here we describe a novel approach for design of recombinant Envs that not only ensures the stabilization of the gp120−gp41 complex but also provides a tool for elucidation of the nature of intermediate Env structures that may have potential as vaccine immunogens and entry inhibitors. We hypothesized that some of the transient Env conformations on the pathway to viral fusion can be exhibited and retained in fusion proteins of gp120 and gp41 ectodomains joined by flexible linkers. The flexible linkers of varying length not only would prevent the dissociation of the noncovalently bound gp120 and gp41 but also would impose structural constraints on the receptor-induced Env conformational changes thus "freezing" them at different intermediates in dependence on the length of the linker. We found that gp120-linker-gp41 proteins (tethered Envs; gp140s) can be stably expressed and exhibit potent inhibitory activity of fusion and entry mediated by Envs from a variety of HIV-1 isolates. The inhibitory mechanism involves exposed conserved gp41 structures capable of interfering with the entry process.

MATERIALS AND METHODS

Cells, Viruses, Plasmids, Soluble CD4, gp120, gp140 Antibodies and Peptides. 3T3 cells expressing CD4 and CCR5 were gift from D. Littman (New York University, NY, NY). Cf2Th cells expressing high amounts of CCR5 were gift from J. Sodroski (Dana Farber Institute, Boston, MA); the parental cells was purchased from ATCC and used as negative control. The stable cell line TF228 expressing LAI Env was a gift from Z. Jonak (SmithKline Beechman Pharmaceuticals, Philadelphia, PA) through R. Blumenthal (NCI−Frederick, Frederick, MD). Recombinant vaccinia viruses used for the reporter gene fusion assay were described previously (*31*). Plasmids expressing various Envs were obtained through the NIH AIDS Research and Reference Reagent Program or were gifts from R. Doms (University of Pennsylvania, Philadelphia, PA) and G. Quinnan (Uniformed Services University, Bethesda, MD). The expression vector, pEF1/His was purchased from Invitrogen. Two-domain soluble CD4 (sCD4) was a gift from E. Berger (NIAID, Bethesda, MD). Purified $gp120_{89.6}$ and $gp140_{89.6}$ were produced by recombinant vaccinia virus (gift of R. Doms (University of Pennsylvania, Philadelphia, PA)) with a combination of lentil lectin affinity chromatography and size exclusion chromatography. The anti-CD4 polyclonal antibody T4-4 was obtained through the AIDS Research and Reference Reagent Program from R. Sweet (SmithKline Beechman Pharmaceuticals, Philadelphia, PA). The anti-gp41 mAbs 50-69, 246-D, 98-6, 126-6, and 240-D were obtained through the NIH AIDS Research and Reference Reagent Program from S. Zolla-Pazner (New York University, NY, NY) and Md-1 from R. Myers (State of Maryland, DHMH, Laboratories Administration, Baltimore, MD), the anti-CCR5 mAb 5C7 was a gift from L. Wu (Millenium Pharmaceuticals, Cambridge, MA). The anti-gp120 mAb D19, D25, T36 and M12, and the rabbit polyclonal antibody R2143 as well as the anti-gp41 mAbs D17, D40, D47, D54, D61, T3, and T30 were previously described (23;32). The gp41 peptides DP178, DP107, and C34 were a gift from R. Blumenthal.

Production, Purification, and Quantification of gp120-linker-gp41 Fusion Proteins. Fusion proteins were expressed from transiently transfected 293T cells by using Polyfect (Qiagen Inc., Valencia, CA) according to manufacturer's protocol. Supernatant from transiently transfected cells was collected 48 h after transfection and analyzed for protein expression by anti-gp120 and anti-gp41 Abs by Western blotting. We also developed stable transfectants that had been adapted for growth in 293-serum free medium (Gibcol-BRL, Gaithersburg, MD) and constitutively express the fusion proteins. The proteins were further purified by using lentil lectin Sepharose 4B affinity chromatography (Amersham-Pharmacia Biotech). Bound protein was eluted from the lectin column by 1 M methyl-α-D-mannopyranoside. The eluted protein was then dialyzed against PBS and concentrated by Millipore concentrator. The protein concentrations were determined by BCA assay (Pierce, CA) according to manufacturer's protocol. For accurate quantification of the fusion proteins, they were run on a 10% SDS−PAGE gel simultaneously with calibrating amounts (1, 3, 10, 30, 100 ng) of highly purified gp140 and were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 20 mM tris-HCl (pH 7.6) buffer containing 140 mM NaCl, 0.1% Tween-20, and 5% nonfat powdered milk. For Western blotting, these membranes were incubated with anti-gp120 antibodies, then washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. They were developed by using the supersignal chemiluminescent substrate from Pierce (Rockford, Il). The images were acquired using a BioRad phosphoimager (BioRad, Hercules, CA). The signal from the calibrating molecules was integrated for each band and plotted in a calibration curve for the signal vs amount dependence. The amounts of fusion proteins were then calculated by interpolation using the calibration curve.

Size Exclusion Chromatography. The fusion proteins were analyzed under nondenaturing conditions by gel filtration chromatography on a preparative superdex200 column (Amersham-Pharmacia Biotech). The column was equilibrated with PBS, calibrated, and then standardized using high molecular weight gel filtration kit (Amersham-Pharmacia Biotech) with protein standards ranging from 158 to 669 kDa. A standard curve of elution volume versus molecular weight was then generated. Regression analyses based on the curve was then used to estimate the molecular weights of tested proteins run on the column. Samples of the fusion proteins were applied to the column in 1 mL of PBS. The column was run at a constant flow rate of approximately 1.1 mL/min, and washed with PBS at identical flow rate; fractions were collected in 120 mL. The fusion proteins were assigned by using the highest peak or shoulder in a chromatogram as the basis for making calculation and then analyzed by immunoblot assay with anti-gp41 antibodies, and enzyme-linked immunosorbent assay (ELISA).

ELISA Binding Assay. The assay used for binding of purified molecules was a modified ELISA type assay. The test proteins, e.g., sCD4, were nonspecifically attached to the bottom of 96-well plates by incubation of 0.1 mL solution containing 100 ng of the protein at 4 °C overnight. Plates were then treated with PBS containing 2% BSA and 0.5% Tween-20 (PBS−BSA−Tween) to prevent nonspecific binding. The plates were washed with TBS, test samples were diluted in PBS−BSA−Tween and incubated for 1 h at room temperature. All antibodies were diluted in PBS−BSA−Tween and plates were washed five times with TBS between each incubation step. Bound antigen was detected using a

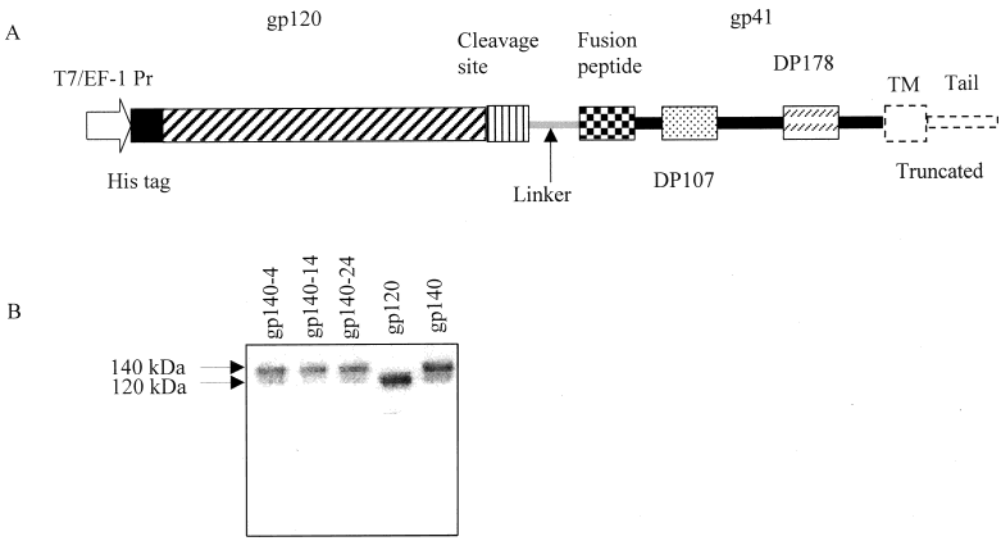

FIGURE 1: Schematic representation (not in scale) of tethered HIV-1 gp140 fusion proteins (gp140-4,14,24) (A) and their expression detected by Western blotting (B). (A) The postranslational cleavage site was mutated and linkers of 4 (GILI), 15 (GILQISSSGGAGGK), or 26 (GILQISGSGSGGSGQGSSGGAGGK) amino acid residues were introduced for the gp140-4, 14, or 24 constructs, respectively. For all constructs, the transmembrane (TM) domain and the cytoplasmic tail of gp41 were truncated. The fusion peptide, and the DP107 and DP178 sites in gp41 are also indicated. (B) 293T cells were transiently transfected with plasmids encoding tethered gp140-4,14,24. Supernatant was collected after 48 h and subjected to SDS−PAGE electrophoresis. Recombinant $gp120_{89.6}$ and $gp140_{89.6}$ produced by vaccinia virus recombinants were used as controls. An anti-gp120 antibody (D19) was used for Western blotting.

pool of anti-gp41 Abs as described above followed by the appropriate labeled secondary antibody. The amount of bound fusion proteins was determined based on a standard curve generated with purified 89.6 gp140. Biotinylated proteins for use in this assay were prepared by incubation with 2 mM biotin (prepared from solid NHS−LS−Biotin (Pierce, CA) dissolved at 200 mM in DMSO as stock solution) on wet ice for 1 h. The biotinylation was quenched with 20 mM glycine on ice for 15 min.

Flow Cytometry Cell Surface Binding Assay. Binding of the fusion proteins to cell surface associated proteins was evaluated by using flow cytometry as previously described (*33*). Cells (typically 0.5×10^6) were incubated for 1 h on ice with the fusion proteins and sCD4, then washed and incubated with gp120-, CD4-, or CCR5-specific antibodies at 1 μg/mL. They were again washed, and incubated for another hour on ice with rabbit IgG (10 μg/mL) (Sigma, St. Louis, MO) to improve the specificity, then washed and incubated for 1 h with an anti-mouse phycoerythrin-conjugated polyclonal antibody or anti-rabbit FITC-conjugated polyclonal antibody for gp120 and CD4 (Sigma). The cells were washed and fixed with paraformaldehyde on ice for 10 min. The flow cytometry measurements were performed with FACSCalibur (Becton Dickinson, San Jose, CA).

Cell−Cell Fusion. The cell−cell fusion assay was previously described (*31*). Briefly, recombinant vaccinia viruses at multiplicity of infection 10 were used to infect the target (vCB21R) and effector cells (vTF 7.3). The β-gal fusion assay was performed 2 h after mixing the cells. The extent of fusion was quantitated colorimetrically. Inhibition of cell−cell fusion was also quantitated by using a syncytium assay where cells expressing Env were mixed with equal number of cells expressing CD4 and coreceptor molecules, and the number of syncytia was counted 4 h later.

HIV-1 Entry. Evaluation of HIV-1 entry inhibition was performed by using infection with a luciferase reporter HIV-1 Env pseudotyping system (*34*). Viral stocks were prepared by transfecting 293T cells with plasmids encoding the luciferase virus backbone (pNL-Luc-ER) and Env from various HIV strains. The resulting supernatant was clarified by centrifugation for 10 min at 2000 rpm in a Sorvall RT-7 centrifuge (RTH-750 rotor) and stored at 4 °C. The virus was preincubated with various concentrations of inhibitors for 1 h at 37 °C. Cells were then infected with 100 μL of virus preparation containing DEAE-dextran (8 μg/mL) for 4 h at 37 °C. After five washes with PBS, fresh medium (0.2 mL) was added to each well in a 96-well plate. Cells were lysed 44 h later by resuspension in 100 μL of cell lysis buffer (Promega, Madison, Wis.), and 50 μL of the resulting lysate was assayed for luciferase activity, using an equal volume of luciferase substrate (Promega).

RESULTS

Design, Production, and Purification of gp120-linker-gp41 Proteins (Tethered Env). To produce stable fusion proteins between gp120 and gp41 joined by flexible linkers we mutated the proteolytic cleavage site (Figure 1A) of the envelope glycoprotein (Env) derived from the primary R5X4 HIV-1 isolate 89.6 (*35*). Three different fusion proteins designated as gp140-4, gp140-14, and gp140-24 were developed where gp120 and gp41 are joined by linkers of 4, 15, and 26 amino acid residues long, respectively. Stop codons were introduced into the *env* gene (GenBank accession numbers U39362, AAA81043), resulting in termination of the proteins N-terminal to the transmembrane domain of gp41. Thus, the fusion proteins do not contain the transmembrane domain and cytoplasmic tail of gp41, and are secreted in the medium of the expressing cells (Figure 1A). Stable cell lines were developed by transfection of 293T cells with plasmids encoding the fusion proteins. The culture supernatants were collected, and the fusion proteins were purified by lentil lectin and size exclusion chromatography.

Characterization of the gp140 Fusion Proteins. The amount of the tethered proteins was quantified from Western

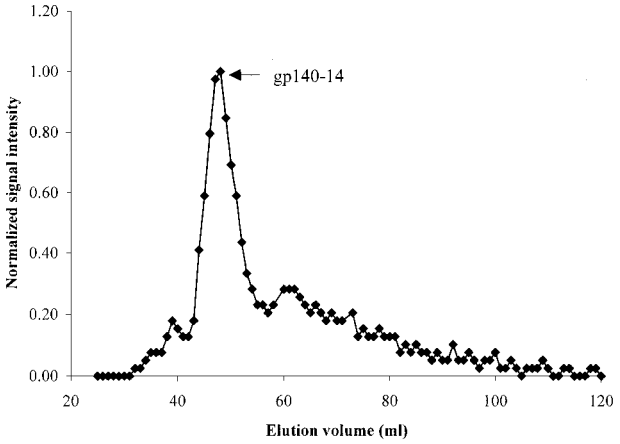

FIGURE 2: Size exclusion chromatography analysis of tethered gp140-14. Chromatography was carried out on a Superdex 200 column as described in the Materials and Methods. Fractions were collected from the column and analyzed by ELISA using anti-gp41 antibodies and by Western blotting using anti-gp120 antibodies.

Table 1: Binding of gp140-14 Complexed with Two-Domain sCD4 to Cell Surface Associated CCR5[a]

antibody to	no ligand	sCD4	gp140-14	gp140-14 + sCD4	gp140 + sCD4
CCR5	1.1×10^3	1.3×10^3	1.2×10^3	1.1×10^3	370
CD4	0.1	20	1	8	7
gp120	0	0.02	1.3	20	5

[a] CF2Th-CCR5 cells were incubated with gp140-14, sCD4, gp140-14-sCD4, gp140-sCD4 at 5 μg/mL (except sCD4 which was at 1 μg/mL) or without ligands at 4 °C for 1 h. After five washes, cell surface binding was tested by anti-CC5 mAb (5C7), anti-CD4 polyclonal antibody (T4-4), and an anti-gp140 polyclonal antibody (R2143) using flow cytometry as described in Materials and Methods. The background binding was measured by using the secondary antibody in the absence of the specific antibody and subtracted. The binding is represented as the geometric mean of fluorescence intensity in arbitrary units.

blots by using phosphoimager and calibrating curves from serial dilutions of purified $gp140_{89.6}$ with known concentrations produced by recombinant vaccinia virus. The concentration in the culture supernatants was about 5 μg/mL, and after purification it was increased to 0.7 mg/mL. The culture supernatants from the transfectants producing the fusion proteins and purified proteins were tested by Western blotting. As expected, the molecular weight (MW) of the fusion proteins on SDS−PAGE was close to 140 kDa (Figure 1B). There was a minor lower MW band containing less than 10% gp120 probably due to the use of second inefficient cleavage site (Figure 1B). Size exclusion chromatography of the purified proteins revealed that they are predominantly monomeric with a very low concentration of dimers and gp120 (Figure 2). The tethered proteins complexed with two-domain soluble CD4 (sCD4) bound cell surface-associated CCR5 similarly to uncleaved gp140 complexed with sCD4 (Table 1). There was no binding of sCD4, gp140-14-sCD4, or the anti-CCR5 mAb 5C7 to the parental cell line Cf2Th (data not shown). These data suggest that the expressed fusion proteins are able to interact with receptors involved in HIV-1 entry.

The native conformation of the tethered proteins was also tested by ELISA using conformationally dependent anti-gp120 (M12, X5, and D25) and anti-gp41 (D54) mAbs. There were no significant differences in the binding of these

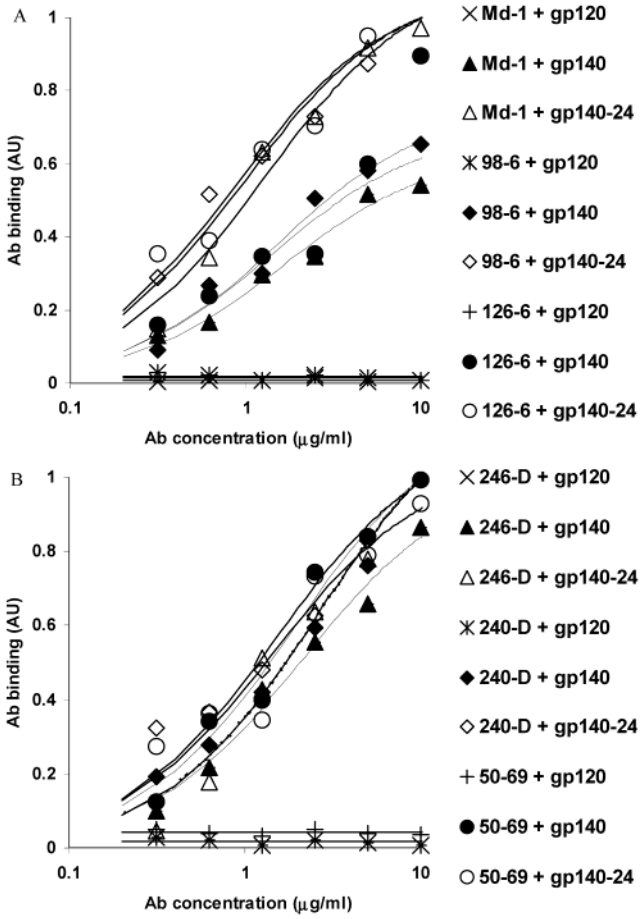

FIGURE 3: Enhanced exposure of gp41 structures in gp140-24 (long linker) compared to gp140 (no linker). Serial (2-fold) dilutions of the antibodies were applied to the proteins and the amount of bound antibodies measured by an ELISA assay as described in Materials and Methods. The background was estimated by the amount of antibody bound to BSA and subtracted; gp120 was used as negative control. The data were fitted (continuous lines) to the Langmuir adsorption isotherm ($B/B_{max} = Ab/(K_d + Ab)$), where B is the amount of bound Ab, B_{max} is the maximal amount of bound Ab, Ab is its bulk concentration, and K_d is the equilibrium dissociation constant. The data are normalized to the highest OD values for the respective antibody assumed to be 1. (A) Enhanced binding of gp41 cluster I mAb (98-6, 126-6, and Md-1): K_d(gp140-24)/K_d(gp140) = 0.58, 0.69, 0.80, and B_{max}(gp140-24)/B_{max}(gp140) = 1.43, 1.56, 1.76, respectively. (B) Lack of enhanced binding for gp41 cluster II mAb (50−69, 246-D, and 240-D): K_d(gp140-24)/K_d(gp140) = 0.59, 0.75, 1.21, and B_{max}(gp140-24)/B_{max}(gp140) = 0.91, 0.88, 1.23, respectively.

antibodies to gp140-4,14,24 compared to uncleaved gp140 (data not shown). These data suggest that the tethered proteins are likely to be antigenically similar to uncleaved Envs except for some gp41 structures as described below.

Enhanced Exposure of gp41 Structures. To evaluate the extent of enhanced exposure of various gp41 epitopes in the tethered proteins, we used a battery of anti-gp41 mAbs. We found that certain mAbs as 98-6, 126-6 (cluster II), and Md-1 bound better to gp140-24 than to gp140 (Figure 3A). Therefore, their epitopes that overlap or include the DP178 site are better exposed. However, other antibodies as 50-69, 246-D, and 240-D (cluster I) with epitopes overlapping the region including the C-terminal part of DP107 and immediately downstream of the DP107 site did not exhibit enhanced binding (Figure 3B). We did not find enhanced

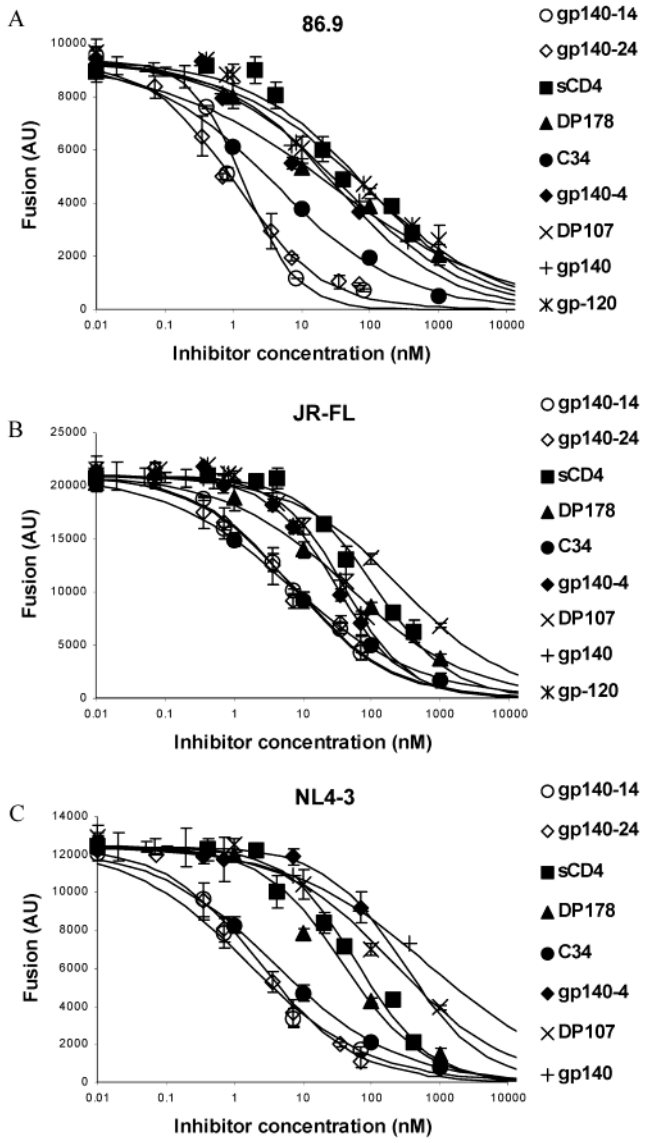

FIGURE 4: Inhibition of virus entry by gp140-14 and gp140-24 in comparison with known inhibitors and controls. HIV-1 entry inhibition was quantified by using a luciferase reporter HIV-1 Env pseudotyping system as described in Materials and Methods. The extent of lucipherase activity in the absence of inhibitor was the same as in the presence of any inhibitor at 0.1 nM and in the presence of DP107 at 1 nM. The data were fitted to the function $OD/OD_{max} = I^n/(IC_{50}{}^n + I^n)$, where OD is optical density, OD_{max} is its maximal value, I is bulk concentration of the inhibitor, n is constant, and IC_{50} is 50% inhibitory concentration. (A) Infection of HOS CD4.CXCR4 cells by pseudotyped HIV-$1_{89.6}$. $IC_{50} = 1.1$, 1.5, 27, and 30 nM for gp140-24, gp140-14, DP178, and gp140-4, respectively. (B) Infection of HOS CD4.CCR5 cells by pseudotyped HIV-1_{JR-FL}. $IC_{50} = 7.3$, 7.4, 46, and 31 nM for gp140-24, gp140-14, DP178, and gp140-4, respectively. (C) Infection of HOS CD4.CXCR4 cells by pseudotyped HIV-1_{NL4-3}. $IC_{50} = 2.4$, 1.7, 40, and 300 nM for gp140−24, gp140-14, and gp140-4, respectively.

binding to gp140-14,24 of any anti-gp120 mAb (D19, D25, T36, and M12) we tested.

Inhibition of HIV-1 Env-Mediated Membrane Fusion. To find whether the tethered gp140s inhibit Env-mediated membrane fusion, we used a luciferase reporter Env pseudotyping system for virus infection (Figure 4), a β-gal reporter gene and syncytia formation assays for cell fusion (Table 2). For infections of HOS.CD4.CXCR4(CCR5) cells mediated by 89.6, JR-FL, or NL4-3 Envs the 50% inhibitory concentration (IC_{50}) for the two tethered proteins with long linkers (gp140-14 and gp140-24) were on average 5−100-fold lower than those for the tethered protein with short linker (gp140-4), the truncated uncleaved Env without linker (gp140), gp120, DP107, or DP178 (Figure 4). The inhibitory activity of C34 was comparable with gp140-14,24 but only for infection mediated by the JR-FL Env. For 89.6 and NL4-3 Envs the gp140-14,24 IC_{50} was 2−4-fold lower than that of C34. Fusion between TF228 and SupT1 cells was also potently inhibited (Table 2). The cell fusion IC_{50} was in the range from 0.3 to 3 nM. Cell fusion mediated by several X4, R5, and X4R4 Envs was similarly inhibited suggesting independence of the inhibitory mechanism on the coreceptor usage (Figure 5). Note that for the data on Figure 5, the gp140-14 concentration used is 10-fold lower than for gp140-4 and gp140, and that there is background due to nonspecific β-gal activity for cells expressing Envs which bind to CD4 but do not proceed to fusion for lack of appropriate coreceptor. The inhibitory activity of the two long linker fusion proteins, gp140-14 and gp140-24, was about the same but with a trend to higher activity of gp140-24 suggesting that the structures responsible for the inhibitory activity may be better exposed for longer linkers although the linker length effect was not statistically significant for linker lengths in the range from 15 to 26 amino acid residues.

Mechanism of the Specific Inhibitory Activity of the Fusion Proteins with Long Linkers. In an attempt to elucidate the mechanism of the enhanced inhibitory activity of gp140-14,24 compared to gp140-4 we examined the role of the enhanced exposure of gp41 structures by incubating the tethered Envs with anti-gp41 mAbs that do not inhibit Env-mediated fusion. All mAbs that exhibited enhanced binding to the tethered proteins gp140-14,24 reversed the tethered Env inhibitory effect to levels comparable to those exhibited by gp140 (Table 3). Antibodies that bound equally well gp140-14,24 and gp140 did not significantly affect the inhibitory effect of the tethered Envs. These results suggest that the exposed gp41 structures detectable by cluster II mAbs are responsible for the potent inhibitory activity of the tethered Envs. Finally, to further address the proposed mechanism of inhibition, we compared the inhibition by the gp140-linker protein in the presence and absence of DP178 to DP178 alone, gp120 alone, and gp120 in the presence of DP178 all at a concentration of 10 nM. Here, there was an additive effect of gp120 and DP178 to a level of inhibition of cell−cell fusion to 65% compared to the control (no inhibitors added). Whereas, gp140-24 alone inhibited to a level of 84% and gp140-24 + DP178 together yielded an inhibition level of 86% (data not shown).

DISCUSSION

A major new finding of this work is that fusion proteins of gp120 and gp41 joined by flexible linkers equal to or longer than 15 amino acid residues are potent broadly reactive HIV-1 entry inhibitors with IC_{50} in the range from 0.3 to 3 nM in dependence on the experimental system used. Because the IC_{50} of the fusion protein with short (four amino acid residues) linker or without linker is about 100-fold higher than IC_{50} for gp140-14,24 it appears that the binding to target cell-associated CD4 is not the primary mechanism responsible for the high inhibitory activity. The specific reversion of the gp140-14,24 inhibitory effect by cluster II

Table 2: Inhibition of Cell Fusion by gp140-14 and gp140-24[a]

inhibitor conc μg/mL	sCD4 β-gal act. %	gp140 β-gal act. %	gp140-4 β-gal act. %	gp140-24 β-gal act. %	gp140-14 β-gal act. %	gp140-14 Syncytia %
0.01	98 ± 2	102 ± 2	99 ± 4	70 ± 10	77 ± 9	64 ± 5[b]
0.1	95 ± 4	98 ± 3	100 ± 2	24 ± 1	39 ± 1	29 ± 16
1	67 ± 1	100 ± 2	98 ± 1	15 ± 1	22 ± 4	11 ± 6
10	25 ± 2	77 ± 5	76 ± 7	8.2 ± 0.5	14 ± 1	7.4 ± 2.1

[a] 10^5 TF228 cells expressing LAI Env and 10^5 SupT1 cells were preincubated at different concentrations of the inhibitor for 1 h at 37 °C, then mixed together in a 96-well plate and incubated for 2 h at 37 °C followed by measurement of β-gal activity or number of syncytia. The data are presented as percentage of fusion in the absence of inhibitor assumed to be 100% as the mean ± standard deviation of duplicated experiment.[b] At concentration 0.05 μg/mL.

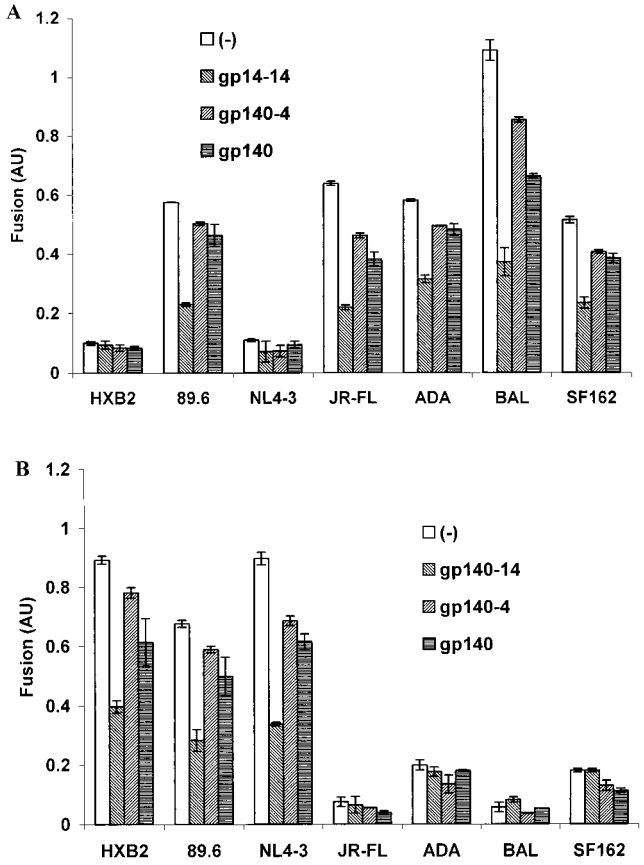

FIGURE 5: Independence of cell fusion inhibition by gp140-14 on coreceptor usage. 293T cells transfected by plasmids encoding various Envs and target cells were preincubated with gp140-14 at 100 ng/mL, gp140-4 or gp140 at 1 μg/mL, for 1 h at 37 °C, then mixed together and incubated for 2 h at 37 °C. The extent of fusion was determined by the β-gal assay as described in Materials and Methods. (A**)** Target cells − SupT1 cells (which express CD4 and CXCR4). **(**B**)** Target cells − NIH 3T3 CD4.CCR5 cells.

mAbs and Md-1 but not by cluster I, IV, and VI mAbs strongly implicates a role for C-terminal gp41 structures overlapping the DP178 sites. One can speculate that these gp41 structures could interact with intermediate structures of the Env mediating HIV-1 entry similarly to DP178. One possible scenario of the tethered protein inhibitory activity includes prevention of the interactions between N-terminal and C-terminal sequences of gp41 leading to formation of fusion intermediates (*4*, *36*). Another possibility is the existence of other structures yet to be identified. Yet, another possible mechanism is a dominant negative interference with the oligomeric structures and/or multimeric fusion complexes. One cannot also exclude possible synergistic effects

Table 3: Reversion of the gp140-14,24 Inhibitory Effect by Cluster II mAbs and Md-1 but Not by Cluster I, IV, and VI mAbs[a]

Ab	recognition site	reversion of inhibitory effect (%) gp140-24	gp140-14
Md-1	aa 565−685	73	74
D61	cluster I	4.9	ND
246-D	cluster I aa 579−604	16	18
50−69	cluster I aa 579−613	8.7	12
240-D	cluster I aa 579−604	0.6	3.7
D17	cluster II	56	ND
D40	cluster II	46	ND
98−6	cluster II aa 644−663	72	61
126−6	cluster II aa 644−663	64	51
T3	cluster IV	8.2	ND
T30	cluster VI	13	ND

[a] The tethered proteins (1 μg/mL) were mixed with the mAbs (10 μg/mL) for 30 min at 37 °C, the mixture was preincubated separately with the effector (TF228) and target (SupT1) cells for another 30 min after which they were mixed for 2 h at 37 °C, and the β-gal activity was measured as described in Materials and Methods. The percentage of inhibitory effect reversion (%) was calculated by subtracting the percentage of fusion inhibition in the presence of antibodies from the percentage of fusion inhibition in the presence of tethered proteins and normalizing to 100% (by dividing with the percentage of fusion inhibition in the presence of tethered proteins and multiplying by 100). ND − not determined.

between mechanisms involving gp41 structures and the binding of gp120 to CD4.

The high functional activity of the tethered Envs (gp140-14,24) provides new opportunities for development of potent inhibitors of HIV-1 entry although in their current form they may not be appropriate for in vivo use because of their immunogenicity and binding to CD4. However, they can be used as a basis for design of appropriate inhibitors for human use including as microbicides. In another avenue of possible applications, the tethered Envs could serve as potent immunogens. The linker serves two purposes. First, it prevents shedding of gp120 from gp41 so the protein will retain its structure close to the native one. Second, because of the relatively large size of the linker some regions of the extracellular portion of gp41 could be exposed and reveal conserved epitope. The conserved nature of these structures is indicated by the high inhibitory effect of the tethered Envs on a variety of HIV-1 isolates. Thus, the tethered Envs could be used as immunogens eliciting broadly cross-reactive antibodies. Although those mAbs (cluster II and Md-1) that bind specifically to parts of these structures are not neutralizing or only weakly neutralizing one can envision that regions of these structures in the native Env environment could elicit neutralizing antibodies that have not been identified yet. Immunizations of animals and screening of

phage display libraries are now in progress to further explore this possibility. We have recently established that these tethered gp140 proteins can be recognized by several of the most potently and broadly neutralizing antibodies known, including 2G12 and IgG-b12 and a novel human monoclonal Fab, X5 (*37*). Finally, these fusion proteins could prove invaluable in dissecting the transient conformational changes of the Env on the pathway to fusion. These conformational changes are rapid and difficult to analyze. It is of interest that the linker proteins developed here present as monomeric molecules, which is similar to the recently developed SOS disulfide-linked cleaved gp140 by Moore and colleagues (*28*). The SOS gp140 is also proposed to be more closely relevant to native Env structure and perhaps more flexible, yet upon further analysis of this protein it has been shown to be monomeric as well (*38*). It is perhaps the added flexibility imposed by linkers or allowing gp120−gp41 cleavage in a linked molecule that induces oligomer dissociation. The novel approach of imposing constraints by using flexible linkers of varying length can lead to stabilization of the transient intermediate structures that would allow their detailed investigation. Such an approach could be used in any other system where rapid transient conformational changes of proteins are difficult to study. Whether it will be successful will depend on the particular system under investigation.

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