

# Disulfide Bond That Constrains the HIV-1 gp120 V3 Domain Is Cleaved by Thioredoxin\*

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A functional disulfide bond in both the HIV envelope glycoprotein, gp120, and its immune cell receptor, CD4, is involved in viral entry, and compounds that block cleavage of the disulfide bond in these proteins inhibit HIV entry and infection. The disulfide bonds in both proteins are cleaved at the cell surface by the small redox protein, thioredoxin. The target gp120 disulfide and its mechanism of cleavage were determined using a thioredoxin kinetic trapping mutant and mass spectrometry. A single disulfide bond was cleaved in isolated and cell surface gp120, but not the gp160 precursor, and the extent of the reaction was enhanced when gp120 was bound to CD4. The Cys<sup>32</sup> sulfur ion of thioredoxin attacks the Cys<sup>296</sup> sulfur ion of the gp120 V3 domain Cys<sup>296</sup>-Cys<sup>331</sup> disulfide bond, cleaving the bond. Considering that V3 sequences largely determine the chemokine receptor preference of HIV, we propose that cleavage of the V3 domain disulfide, which is facilitated by CD4 binding, regulates chemokine receptor binding. There are 20 possible disulfide bond configurations, and, notably, the V3 domain disulfide has the same unusual –RHStaple configuration as the functional disulfide bond cleaved in CD4.

The human immunodeficiency virus (HIV) is the agent responsible for AIDS. The HIV envelope glycoprotein (env)<sup>2</sup> is translated as a single polypeptide chain (gp160) that is proteolytically cleaved by host cell subtilisins into two noncovalently associated fragments, the surface glycoprotein subunit (gp120) and the transmembrane (gp41) subunit that is anchored in the viral membrane (1). env is a trimer of gp41/gp120 heterodimers on the viral surface that is activated by sequential binding to CD4 and chemokine receptor CXCR4 or CCR5 on susceptible cells. gp120 is thought to dissociate from gp41, which allows the fusion peptide to be inserted into the target membrane. The end result is formation of a six-helix bundled gp41 ectodomain that drives the membrane merger and eventual fusion (2). Functional disulfide bonds in both HIV gp120 and immune cell CD4 are involved in viral

entry, and compounds that block cleavage of these disulfide bonds inhibit HIV entry and infection (3–7).

The disulfide bond in the second extracellular immunoglobulin-like domain of CD4, Cys<sup>130</sup>-Cys<sup>159</sup>, is reduced on the T cell surface by thioredoxin, which leads to formation of disulfide-linked homodimers (7–9). Domain swapping of D2 has been identified as the most likely mechanism for formation of the dimer, where the domain-swapped dimer is held together by two disulfide bonds between Cys<sup>130</sup> in one monomer and Cys<sup>159</sup> in the other one (10, 11). Inhibiting CD4 dimer formation by mutating residues in the D1 and D4 domains enhances viral entry (12), whereas blocking dimer formation by ablating the D2 disulfide bond markedly impairs the CD4 immune co-receptor function (10).

Either one or two of the nine disulfide bonds in gp120 is cleaved during viral and target cell membrane fusion (3–6, 13). It has been hypothesized that reduction of the gp120 disulfide bond(s) facilitates unmasking of the gp41 fusion peptide and its insertion into the target cell membrane. The thiol content of gp120 increases from 0.5–1 to 4 mol of thiol/mol gp120 following interaction of gp120 with cells expressing CD4 and CXCR4 (3). This redox change in gp120 is independent of the chemokine receptor usage of the virus (4). Cleavage of the gp120 disulfide bond is critical for env-mediated cell-cell fusion and HIV entry and infection as these processes are blocked by both small molecule and protein inhibitors of cell surface oxidoreductases (3–6). The oxidoreductase that has received the most attention is protein disulfide isomerase (14). Protein disulfide isomerase co-precipitates with both soluble and cellular CD4 (4), and it has been proposed that gp120 induces assembly of protein disulfide isomerase, CD4, and chemokine receptor into a tetramolecular protein complex serving as a portal for viral entry (5).

The gp120 disulfide(s) can be reduced by protein disulfide isomerase (3, 4), thioredoxin (8), or glutaredoxin-1 (13). This observation implies that the gp120 disulfide bond(s) must have a high redox potential, as protein disulfide isomerase is a poor reductant with a standard redox potential of –175 mV (15). A high redox potential implies that the gp120 disulfide(s) is primed for reduction. Thioredoxin, a small disulfide bond reductant secreted by a variety of cells including CD4<sup>+</sup> cells (7, 16), has been reported to be a more efficient gp120-reducing agent than protein disulfide isomerase (8).

The question we have asked in this study is what disulfide bond(s) is reduced in gp120 during viral entry. This has been

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<sup>2</sup> The abbreviations used are: env, HIV envelope glycoprotein; BisTris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; sCD4, soluble CD4; Trx, thioredoxin; wt, wild type; HEK, human embryonic kidney.

accomplished by mechanism-based kinetic trapping of the gp120 disulfide bond with thioredoxin.

## EXPERIMENTAL PROCEDURES

**Proteins**—Full-length wild type and C35S mutant human thioredoxin-1 cDNAs in the *Escherichia coli* pQE-60 expression vector were kindly provided by Dr. Tobias Dick, German Cancer Research Center, Heidelberg, Germany. The recombinant wild type human thioredoxin-1 (wtTrx) or C35S mutant (C35STrx) proteins were produced as described (9, 17).

The following HIV gp120 proteins were obtained through the National Institutes of Health AIDS Reference and Reagent Program: HIV-1<sub>Bal</sub> gp120 (laboratory isolate), HIV-1<sub>CN54</sub> gp120 (Clade C), HIV-1<sub>93TH975</sub> gp120 (Clade E), HIV-1<sub>96ZM651</sub> gp120 (Clade C). The laboratory isolate was expressed in human embryonic kidney (HEK) 293 cells, and the primary isolates were expressed in insect cells. A recombinant soluble fragment of CD4 containing the four immunoglobulin-like domains (sCD4) was provided by the NIH AIDS Research and Reference Reagent Program.

**Mechanism-based Kinetic Trapping of Purified gp120 Protein with Human Thioredoxin**—wtTrx or C35STrx (4  $\mu$ M) was incubated with HIV-1<sub>Bal</sub> gp120 (4  $\mu$ M) in 0.1 M Na<sub>2</sub>PO<sub>4</sub> buffer, pH 7.0, containing 0.1 mM EDTA for 1 h at 25 °C. On some occasions, incubations were in the presence of sCD4 (4  $\mu$ M). Samples of the reactions were resolved on NuPAGE Novex 4–12% BisTris gel (Invitrogen) with MOPS running buffer under nonreducing conditions and transferred to polyvinylidene difluoride membrane. Proteins were detected by Western blotting using either 1  $\mu$ g·ml<sup>-1</sup> anti-human thioredoxin monoclonal antibody (BD Pharmingen) or 1  $\mu$ g·ml<sup>-1</sup> anti-gp120/160 rabbit polyclonal antibody (Immune Technology) and a 1:1,000 dilution of rabbit anti-mouse or swine anti-rabbit peroxidase-conjugated antibodies (Dako, Carpinteria, CA). Chemiluminescence films were analyzed using a GS-700 Imaging Densitometer and Multi-Analyst software (Bio-Rad).

**ELISA for Reduction of gp120 by Thioredoxin**—HIV-1<sub>Bal</sub> gp120 (100  $\mu$ l of 2  $\mu$ g·ml<sup>-1</sup> in 0.1 M NaHCO<sub>3</sub>, pH 9.6 buffer) was adsorbed to Maxi-Sorp 96-well plates (Nunc, Roskilde, Denmark) for 60 min, and the wells were blocked with 5% skim milk in phosphate-buffered saline (PBS) containing 0.2% Tween 20 for 60 min at 37 °C. wtTrx (4  $\mu$ M in PBS/Tween) was added to wells and incubated for discrete times at 25 °C. The unpaired cysteine thiols in gp120 were alkylated by incubation with 2  $\mu$ M 3-(*N*-maleimidylpropionyl)biocytin (Molecular Probes, Eugene, OR) for 30 min at 25 °C. The wells were washed four times with PBS/Tween and incubated with 100  $\mu$ l of 1:1,000 dilution of StreptABComplex/HRP (Dako) in PBS/Tween for 30 min at 25 °C with orbital shaking. Wells were washed three times, and the bound peroxidase was detected as described previously (18).

**Mechanism-based Kinetic Trapping of Cell Surface gp120 Protein with Human Thioredoxin**—The T4-pMV7 plasmid which contains full-length human CD4 cDNA was provided by the National Institutes of Health AIDS Research and Reference Reagent Program, and the mammalian expression vector pcDNA3 was from Invitrogen. A 3-kb CD4 cDNA was extracted from the T4-pMV7 plasmid as an *EcoRI* fragment and

subcloned into the pcDNA3 vector to produce pcDNA3-CD4. HEK 293T (HEK) cells were transfected with 0.25  $\mu$ g of *env* vector (p89.6 from R. G. Collman, University of Pennsylvania School of Medicine, Philadelphia, PA through the National Institutes of Health AIDS Reference and Reagent Program) (19) or 0.25  $\mu$ g of pcDNA3-CD4 using FuGENE 6 (Roche Applied Science) and incubated for 24 h at 37 °C and 5% CO<sub>2</sub>. The transfected cells were washed twice in serum-free medium, harvested with PBS containing 0.05% EDTA, and resuspended in 1.5 ml of ice-cold PBS. The *env*-transfected cells (0.5  $\times$  10<sup>6</sup> cells) were incubated with C35STrx (4  $\mu$ M) for 1 h at 37 °C, and the reaction was quenched with ice-cold PBS containing 5 mM iodoacetamide. The cells were washed with ice-cold PBS containing 10 mM *N*-ethylmaleimide, and pellets were lysed at 4 °C in 0.5 ml of 50 mM Tris-HCl, pH 8.0, buffer containing 0.5 M NaCl, 1% Triton X-100, 10  $\mu$ M leupeptin, 10  $\mu$ M aprotinin, 2 mM phenylmethylsulfonyl fluoride, and 5 mM EDTA. On some occasions, *env*-transfected cells were co-cultured with CD4-expressing cells for 1 h at 37 °C and 5% CO<sub>2</sub> before addition of C35STrx. The lysate was clarified by centrifugation at 12,000  $\times$  g for 30 min at 4 °C, and samples were resolved on SDS-PAGE and Western blotted as described above.

**Cell-Cell Fusion Assay**—Luciferase reporter assays for cell-to-cell fusion were performed as described (20) with minor modifications. HEK target cells were co-transfected with 0.25  $\mu$ g pTMLuc (a bacteriophage T7 promoter-driven firefly luciferase expression vector (21), 0.25  $\mu$ g of pc.CXCR4 (22), and 0.25  $\mu$ g of pcDNA3-CD4 and incubated for 24 h at 37 °C and 5% CO<sub>2</sub>. HEK effector cells were co-transfected with 0.25  $\mu$ g of *env* vector p89.6 and 0.125  $\mu$ g of pCAGT7 (a T7 RNA polymerase expression vector) (23) and incubated for 48 h at 37 °C and 5% CO<sub>2</sub>. The transfected target cells were washed twice in serum-free medium, harvested with PBS containing 0.05% EDTA, resuspended in 1 ml of complete medium, plated onto effector cells that had been washed twice with PBS, and co-cultured for 24 h at 37 °C and 5% CO<sub>2</sub>. The fusion of effector and target cells was determined using the Luciferase Reporter Assay System (Promega) and expressed as relative light units.

**Mutagenesis of HIV-1 gp160**—pcDNA3.1 plasmid containing the *env* region of the HIV-1 clone 89.6 was provided by Dr. Pantelis Pombourios, Virology Unit, St. Vincent's Institute of Medical Research, Victoria, Australia. The pair of cysteine residues that form the disulfide bond in the V1/V2 stem (Cys<sup>126</sup> and Cys<sup>196</sup>), the V3 domain (Cys<sup>296</sup> and Cys<sup>331</sup>), and the V4 domain (Cys<sup>385</sup> and Cys<sup>418</sup>) were individually mutated to alanine residues. Site-directed mutagenesis was performed using the QuikChange Site-directed Mutagenesis kit (Integrated Sciences, La Jolla, CA) following the manufacturer's instructions. All primers were purchased from Sigma: C126A, 5'-GTAAATTAACCCCACTCGCCGTTACTT-TAAATTG3'-; C196A, 5'-GGTTAATAAGTGCCAACAC-CTCAGTC-3'; C296A, 5'-CTGTAGTAATTAATGCCA-CAAGACCAACAAC-3'; C331A, 5'-GATATAA-GACAAGCACATGCCAACATTAGTAGAGC-3'; C385A, 5'-GAATTTTCTACGCCAATACAGCAC-3'; and C418A, 5'-CACACTCCAAGCCAGAATAAAAC3'-'. All mutations

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were confirmed by automatic sequencing (ABI-377 Automatic Sequencer).

**Mass Spectrometry**—HIV-1<sub>Bal</sub> gp120 (4  $\mu$ M) was left untreated or reacted with C35STrx (4  $\mu$ M) in 0.1 M Na<sub>2</sub>PO<sub>4</sub>, pH 7.0, buffer containing 0.1 mM EDTA for 1 h at 25 °C. Unpaired cysteine thiols in the proteins were alkylated with 10 mM iodoacetamide for 30 min at 25 °C in the dark, and the unreacted iodoacetamide was removed from the solution using Zeba spin desalting columns (Thermo Scientific). The gp120 was denatured for 10 min at 100 °C in 50 mM Na<sub>2</sub>PO<sub>4</sub>, pH 7.5, buffer containing 1 M guanidine HCl and 10 mM dithiothreitol. The solution was cooled, and peptide-*N*-glycosidase F (500 units/ml; Sigma-Aldrich) added to remove the gp120 *N*-glycans. The samples were incubated at 37 °C for 5 h, and the reaction was stopped by heating to 100 °C for 5 min. The protein was digested with chymotrypsin (5  $\mu$ g/ml; Roche Applied Science) for 5 h at 25 °C in 50 mM Na<sub>2</sub>PO<sub>4</sub>, pH 7.5, buffer containing 10 mM CaCl<sub>2</sub>, and the reaction was stopped with 10% acetic acid.

Digested peptides were separated by nano-LC using an Ultimate 3000 HPLC and autosampler system (Dionex, Amsterdam, The Netherlands), and samples were loaded onto a fritless nano column (75  $\mu$ m  $\times$  ~10 cm) containing C18 media (5  $\mu$ m, 200 Å Magic, Michrom). Peptides were eluted using a linear gradient of H<sub>2</sub>O:CH<sub>3</sub>CN (98:2, 0.1% formic acid) to H<sub>2</sub>O:CH<sub>3</sub>CN (64:36, 0.1% formic acid) at 250 nL/min over 60 min. Positive ions were generated by electrospray and analyzed in a LTQ FT Ultra (Thermo Electron, Bremen, Germany) mass spectrometer operated in data-dependent tandem MS acquisition mode. Mass spectral data were searched using Mascot (version 2.2; Matrix Science) against the nonredundant database from NCBI (June 3, 2010). Search parameters were: precursor tolerance 10 ppm and product ion tolerances  $\pm$  0.4 Da. Met-O, Cys-carboxyamidomethyl and Asn-deamination were selected as variable modifications with full chymotryptic cleavage of up to three missed cleavages. To determine the extent of alkylated cysteine residues in the gp120-C35STrx complex, the relative ion abundance of peptides containing Cys-carboxyamidomethyl and unalkylated Cys was used. To calculate ion abundance of peptides, extracted ion chromatograms were generated using the XCalibur Qual Browser software (version 2.0.7; Thermo). The area was calculated using the automated peak detection function built into the software.

**Statistical Analysis**—Statistical analyses were performed using GraphPad Prism (GraphPad, San Diego, CA). Results are presented as means  $\pm$  S.D., and tests of statistical significance were two-sided.

## RESULTS

The disulfide bond or bonds cleaved in gp120 by oxidoreductases during fusion of the viral and cell membranes was determined using a thioredoxin trapping mutant, in which the active site cysteine (Cys<sup>35</sup>) that normally resolves the mixed disulfide has been mutated to serine (9, 17). Replacement of Cys<sup>35</sup> with serine stabilizes the covalent intermediate. Wild-type thioredoxin (wtTrx) and C35S trapping mutant (C35STrx) were expressed in *E. coli* and purified to

homogeneity (Fig. 1A). A small amount of thioredoxin dimer, which is inert as a redox catalyst, was present in both preparations.

**Mechanism-based Kinetic Trapping of a gp120 Disulfide Bond with Human Thioredoxin**—Incubation of purified gp120 with wtTrx or C35STrx resulted in a single complex of gp120 with the thioredoxin-trapping mutant but not with wtTrx (Fig. 1B). The complex was identified by blotting with either thioredoxin or gp120 antibodies (Fig. 1B). The size of the complex (~140 kDa) is consistent with reaction of one molecule of C35STrx with one molecule of gp120, indicating that one gp120 disulfide bond has been reduced. As anticipated, the complex is disulfide-linked as it resolved upon reduction with 20 mM dithiothreitol. C35STrx cleaved a single disulfide bond in four different gp120 isoforms; a laboratory isolate (HIV-1<sub>Bal</sub> gp120), two Clade C isoforms (HIV-1<sub>CN54</sub> and HIV-1<sub>96ZM651</sub> gp120), and a Clade E isoform (HIV-1<sub>93TH975</sub> gp120) (Fig. 1C). This result indicates that the gp120 disulfide bond targeted by oxidoreductases is present and available in different isoforms of the protein.

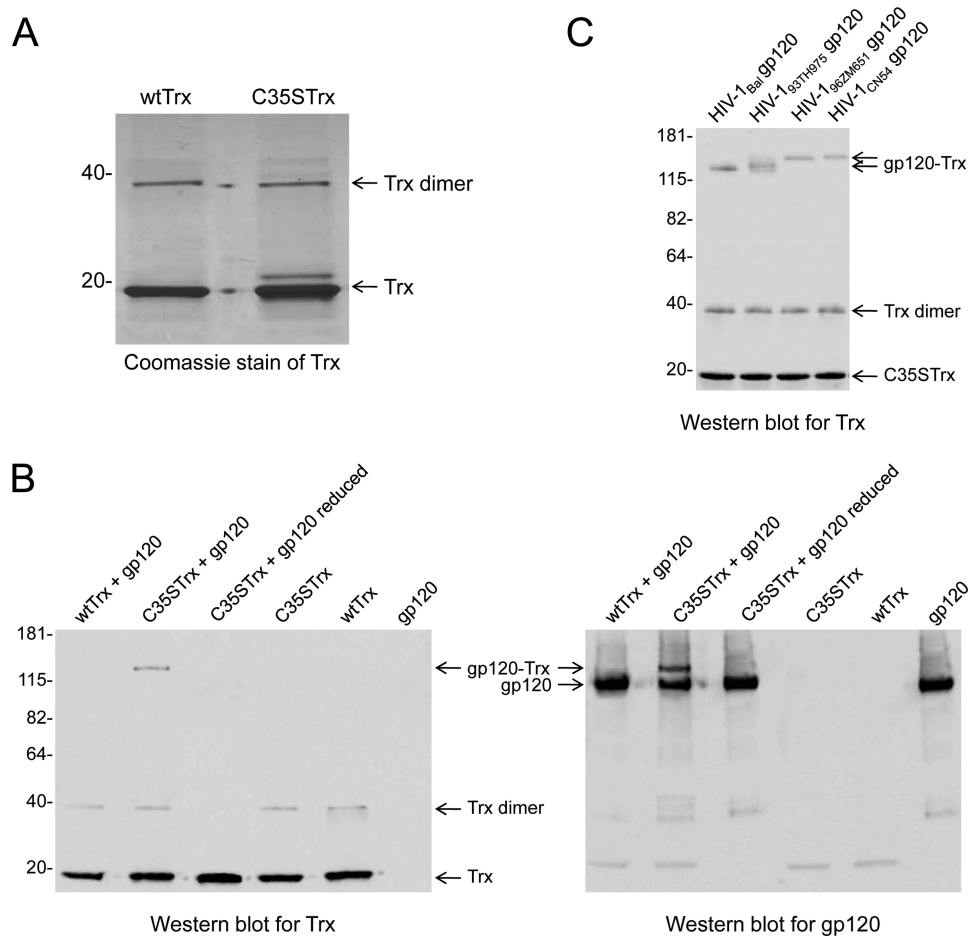
**Binding of gp120 to CD4 Enhances Cleavage of the gp120 Disulfide Bond by Thioredoxin**—Interaction of gp120 with CD4 results in a conformational change in gp120 (24), which suggests that CD4 binding may influence the accessibility or redox potential of the gp120 disulfide bond cleaved by oxidoreductases. This was tested in two different assays, an ELISA that measures reduction of disulfide bond(s) in gp120 and using the C35STrx trapping mutant.

gp120 was coated onto ELISA plate wells and incubated with wtTrx and/or recombinant sCD4 consisting of the four extracellular immunoglobulin domains. Reduction of disulfide bond(s) in gp120 was measured by labeling with a biotin-linked maleimide and detection with avidin-peroxidase. wtTrx reduced one or more disulfide bonds in gp120, which was significantly enhanced by the presence of sCD4 ( $p < 0.001$ ) (Fig. 2A). The assay was specific, as negligible labeling of gp120 was observed in the absence of thioredoxin without or with sCD4. sCD4 also enhanced the reaction of C35STrx with gp120 (Fig. 2B).

**Thioredoxin Cleaves the gp120 Disulfide Bond on the Cell Surface, and Cleavage Is Enhanced by CD4-expressing Cells**—It was important to determine whether the phenomena observed in assays with purified gp120 and sCD4 (Figs. 1 and 2) also occurred on the cell surface with native full-length proteins. HEK cells were transiently transfected with either HIV-1 89.6 *env* or CD4, and reaction of C35STrx with the gp120-expressing cells was measured in the absence or presence of CD4-expressing cells. C35STrx reacted with a single disulfide bond of cell surface gp120, but not with unprocessed gp160, and the reaction was enhanced by the presence of CD4-expressing cells (Fig. 3A). These results parallel those observed using the purified proteins and also indicate that oxidoreductases such as thioredoxin reduce the disulfide bond in gp120 but not its gp160 precursor.

**Reduction of the gp120 Disulfide Bond by Oxidoreductases Such as Thioredoxin Is Not Rate-limiting for *env*-mediated Cell-Cell Fusion**—A feature of HIV infection is syncytium formation between infected cells and CD4<sup>+</sup> cells. HEK target





**FIGURE 1. Mechanism-based kinetic trapping of a gp120 disulfide bond with human thioredoxin.** *A*, wtTrx and C35STrx were expressed in *E. coli* and purified to homogeneity. The proteins were resolved on SDS-PAGE and stained with colloidal Coomassie. *M<sub>r</sub>* markers are shown at left. *B*, wtTrx or C35STrx (4  $\mu$ M) was incubated without or with HIV-1<sub>Bal</sub> gp120 (4  $\mu$ M) for 60 min at 25 °C. Samples of the reactions were resolved on SDS-PAGE under nonreducing or reducing (sample 3 from left) conditions and blotted with anti-Trx (left) or anti-gp120 (right) polyclonal antibodies. Disulfide-linked gp120-Trx complex, gp120, Trx and Trx dimer are indicated. The positions of *M<sub>r</sub>* markers are shown at left. *C*, C35STrx (4  $\mu$ M) was incubated with 4 different gp120 proteins (4  $\mu$ M) for 60 min at 25 °C. Samples of the reactions were resolved on SDS-PAGE under nonreducing conditions and blotted with anti-Trx polyclonal antibodies. Disulfide-linked gp120-Trx complexes, Trx, and Trx dimer are indicated. The positions of *M<sub>r</sub>* markers are shown at left.

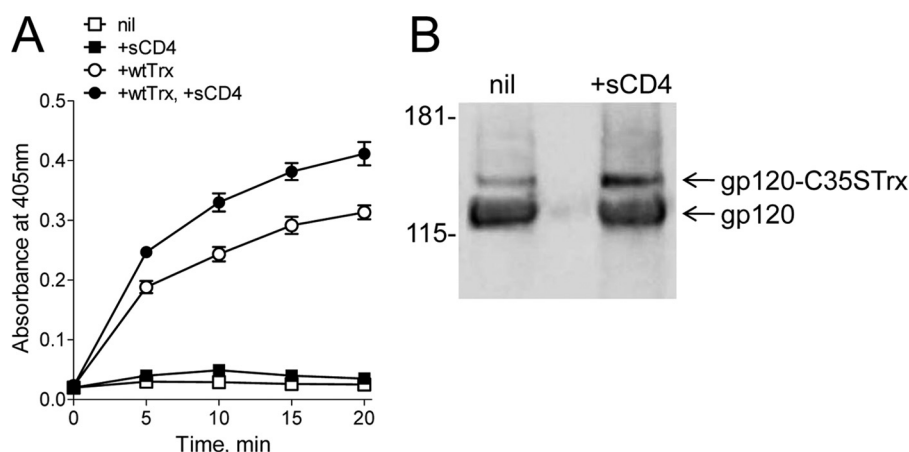
cells were co-transfected with bacteriophage T7 promoter-driven firefly luciferase, CXCR4 chemokine receptor, and CD4. HEK effector cells were co-transfected with HIV-1 89.6 *env* and T7 RNA polymerase. The transfected target cells were plated onto effector cells in the absence or presence of wtTrx or C35STrx and co-cultured for 24 h. Fusion of effector and target cells was determined by luciferase activity. There was no detectable luciferase activity when cells lacking either gp120 or CD4 were used. The presence of micromolar concentrations of the thioredoxins had no significant effect on the extent of cell-cell fusion (Fig. 3*B*). This result suggests that cleavage of the gp120 disulfide bond is not rate-limiting in the cell-cell fusion process. If it were rate-limiting, then addition of exogenous thioredoxin would have been expected to enhance fusion.

**Thioredoxin Cleaves the V3 Domain Cys<sup>296</sup>-Cys<sup>331</sup> Disulfide Bond**—The disulfide bond cleaved in gp120 by C35STrx was determined by mass spectrometry analysis of the gp120-C35STrx complex. The key to the success of this approach was deglycosylation of the gp120 in the complex (Fig. 4*A*). This was required to enable accu-

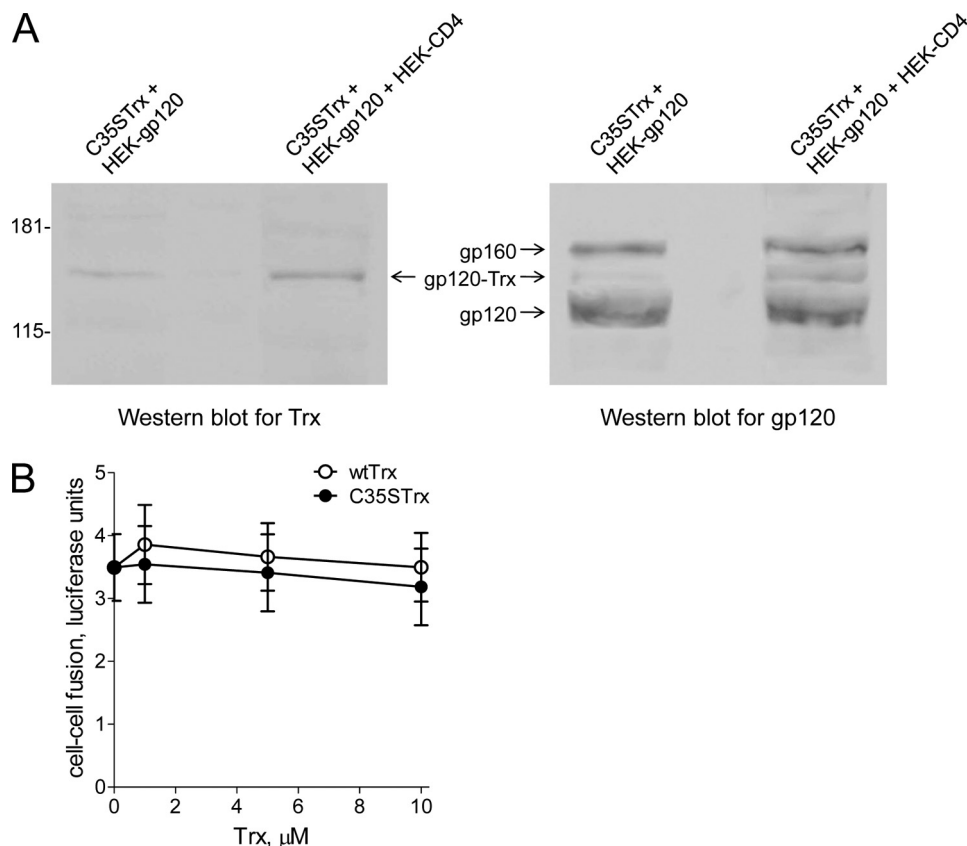
rate prediction of chymotryptic fragment masses. The tandem mass spectrum of the gp120 peptide containing the unpaired (labeled with iodoacetamide) cysteine at position 331 (residues 318–333) is shown in Fig. 4*B*, and the veracity of detection of this peptide is shown in Fig. 4*C*. The 318–333 residue peptide containing unpaired Cys<sup>331</sup> was only and always found in the digest of the gp120-C35STrx complex. It was not observed in digests of gp120 alone. Moreover, no other carboamidomethylcysteinyll-containing peptides were detected in either sample. As controls, the relative abundance of two other unlabeled cysteine-containing gp120 peptides were found to be comparable. This result indicates that the Cys<sup>296</sup>-Cys<sup>331</sup> disulfide bond that constrains the V3 domain of gp120 is cleaved by thioredoxin.

**V3 Domain Disulfide Bond Is Intact in Mature gp160 and Not Cleaved by Thioredoxin**—We sought to confirm that the Cys<sup>296</sup>-Cys<sup>331</sup> disulfide bond was intact in mature gp160 by ablating the bond by mutagenesis and measuring processing to gp120 and trapping with C35STrx. HEK cells were transiently transfected with either wild type or C296A,C331A mu-

## gp120 V3 Domain Disulfide Is Cleaved by Thioredoxin



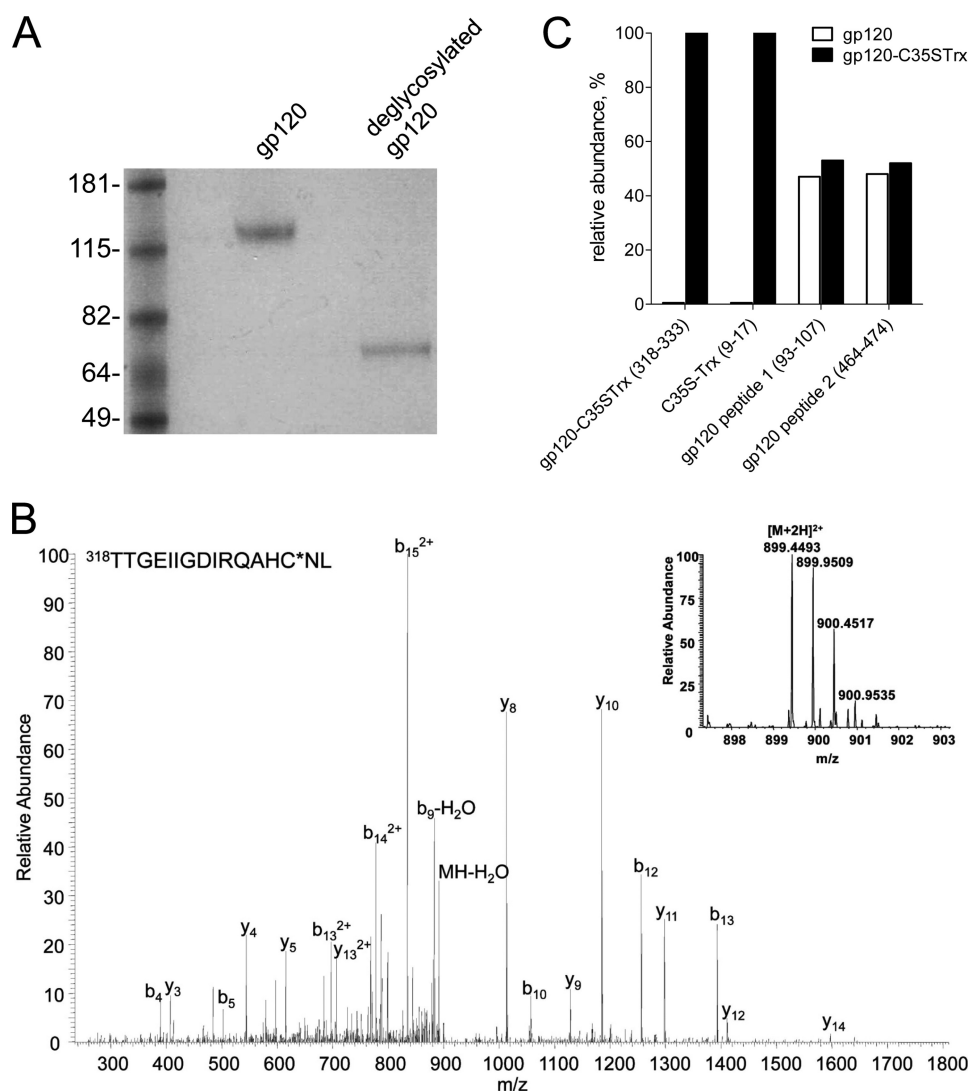
**FIGURE 2. Binding of gp120 to sCD4 enhances cleavage of the gp120 disulfide bond by thioredoxin.** A, HIV-1<sub>Bal</sub> gp120 was coated onto ELISA plate wells and incubated without or with wtTrx (4  $\mu$ M) and/or sCD4 (4  $\mu$ M) for discrete times up to 20 min. The reduced disulfide thiols in gp120 were alkylated with a biotin-linked maleimide, which was then detected using avidin-peroxidase. Trx reduced one or more disulfide bonds in gp120, which was enhanced by sCD4 ( $p < 0.001$ ). The bars and errors are the mean  $\pm$  S.D. of three experiments. B, C35STrx (4  $\mu$ M) was incubated with HIV-1<sub>Bal</sub> gp120 (4  $\mu$ M) in the absence or presence of sCD4 (4  $\mu$ M) for 60 min at 25  $^{\circ}$ C. Samples of the reactions were resolved on SDS-PAGE under nonreducing conditions and blotted with anti-gp120 polyclonal antibodies. Disulfide-linked gp120-Trx complex and gp120 are indicated. The positions of  $M_r$  markers are shown at left. Formation of the gp120-Trx complex was enhanced by the presence of sCD4.



**FIGURE 3. Thioredoxin cleaves the gp120 disulfide bond on the cell surface and cleavage is enhanced by CD4-expressing cells.** A, HEK cells were transiently transfected with either full-length HIV-1 89.6 *env* or CD4. The gp120-expressing cells were incubated with C35STrx (4  $\mu$ M) in the absence or presence of CD4-expressing cells for 60 min at 37  $^{\circ}$ C. The reaction was quenched with iodoacetamide, cell lysates prepared, and samples resolved on SDS-PAGE under nonreducing conditions and blotted with anti-Trx (left) or anti-gp120 (right) polyclonal antibodies. Disulfide-linked gp120-Trx complex, unprocessed gp160, and gp120 are indicated. The positions of  $M_r$  markers are shown at left. Formation of the gp120-Trx complex was enhanced by the presence of CD4-expressing cells. B, effect of wtTrx or C35STrx on the fusion of HEK cells expressing CXCR4 chemokine receptor and CD4 with HEK cells expressing HIV-1 89.6 *env*. Fusion is represented by relative luciferase units. The bars and errors are the mean  $\pm$  S.E. of three separate experiments. There was no fusion of control HEK cells not expressing gp120 or CD4.

tant HIV-1 89.6 *env*. The C126A,C196A and C385A,C418A mutant gp160s were also expressed and tested. The Cys<sup>126</sup>-Cys<sup>196</sup> disulfide bond straddles the V1 and V2 domains,

whereas the Cys<sup>385</sup>-Cys<sup>418</sup> bond constrains the V4 domain. The wild type and C126A,C196A mutant gp160 were processed to gp120 and reacted with C35STrx (Fig. 5). Neither



**FIGURE 4. Thioredoxin cleaves the V3 domain Cys<sup>296</sup>-Cys<sup>331</sup> disulfide bond.** *A*, deglycosylation of gp120 and gp120-C35STrx complex was required to resolve gp120 peptides by mass spectrometry. Native gp120 or gp120 deglycosylated with peptide-*N*-glycosidase F was resolved on SDS-PAGE and stained with colloidal Coomassie. *M<sub>r</sub>* markers are shown at left. *B*, tandem mass spectrum of the gp120 peptide containing an unpaired (labeled with iodoacetamide) cysteine at position 331 (residues 318–333) from the gp120-C35STrx complex. The accurate mass spectrum of the peptide is also shown in the *inset* (observed  $[M+2H]^{2+} = 899.4493$  *m/z*; expected  $[M+2H]^{2+} = 899.4492$  *m/z*). *C*, veracity of detection of the gp120 peptide containing an unpaired Cys<sup>331</sup> (residues 318–333), a C35S-Trx peptide (9–17), and two control gp120 peptides (residues 93–107 and 464–474) in digests of either gp120 (open bars) or gp120-C35STrx complex (filled bars). The gp120 peptide containing unpaired Cys<sup>331</sup> (residues 318–333) and the C35S-Trx peptide (9–17) were only and always found in digests of the gp120-C35STrx complex.

the C296A,C331A nor C385A,C418A gp160 mutant, however, was processed to gp120. These disulfide bonds, therefore, are required for host cell subtilisin processing of gp160. This is presumably due to a requirement for these bonds for correct folding and/or stability of gp160.

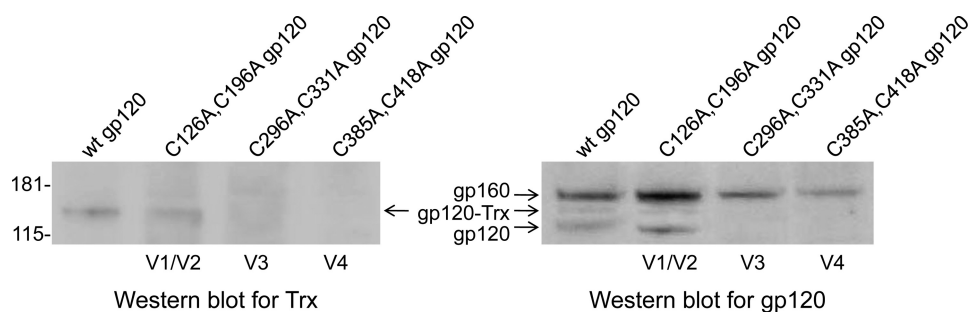
## DISCUSSION

The HIV-1 env complex consists of a trimer of surface-exposed gp120 subunits that are associated with a trimer of gp41 transmembrane subunits. The binding of gp120 to cell surface CD4 leads to the creation of a binding site for a chemokine receptor, usually CCR5 and/or CXCR4. The interaction between gp120 and these cellular receptors initiates conformational changes in the gp120-gp41 complex, triggering the refolding of gp41 into a fusion-active state. gp120 comprises five hypervariable regions (V1–V5) that

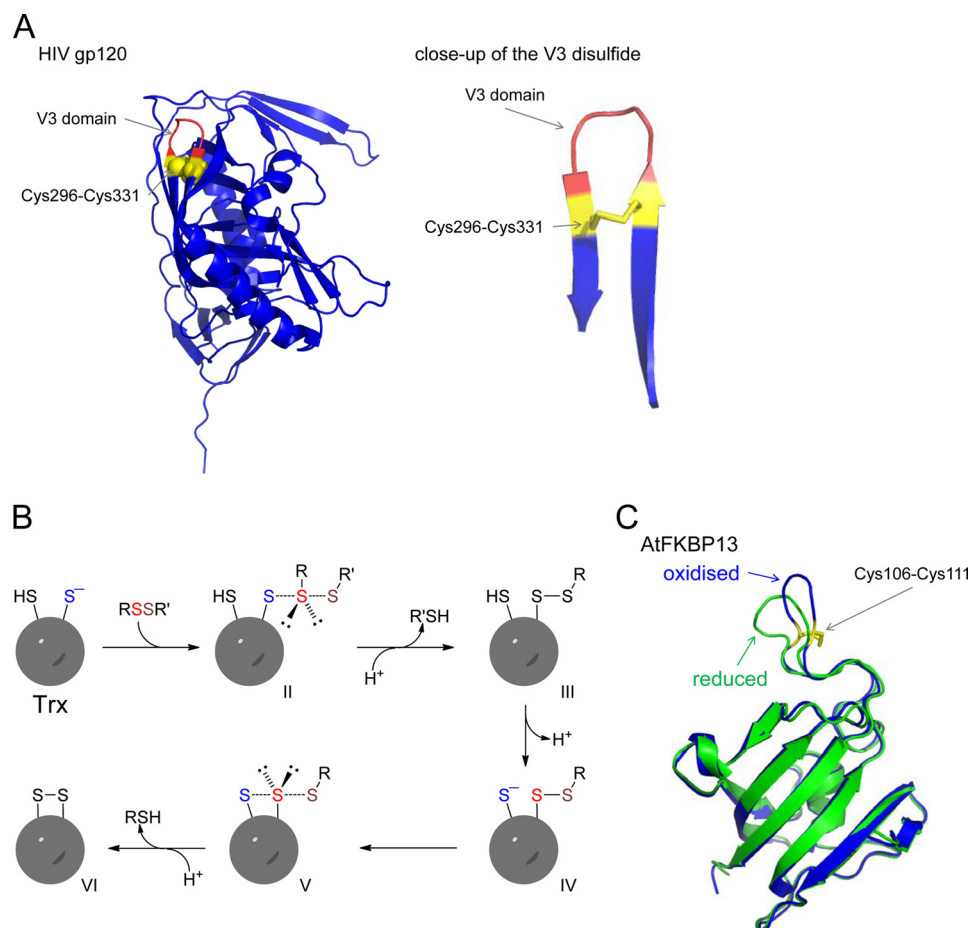
alternate with conserved (C1–C5) regions (25, 26). The gp120 core consists of residues from C1 to C5 forming a conserved inner domain and a less conserved highly glycosylated outer domain. The inner and outer domains are linked through four antiparallel  $\beta$ -strands known as the bridging sheet. The V1–V4 hypervariable regions are excluded from the core domain by disulfide bridges, forming mobile solvent-exposed loops (27).

CD4 and chemokine receptor binding sites are juxtaposed and in close proximity to the bridging sheet, and the V1/V2 and V3 loops. The current understanding is that attachment of CD4 to gp120 leads to a reorganization of the V1/V2 and V3 loops and the formation of the chemokine receptor binding site (25). The chemokine receptor preference of HIV is determined largely by V3 sequences. The CCR5 to CXCR4 switch is generally associated with increased positive charge

## gp120 V3 Domain Disulfide Is Cleaved by Thioredoxin



**FIGURE 5. The V3 domain disulfide bond is intact in mature gp160 and not cleaved by thioredoxin.** HEK cells were transiently transfected with either wild type or C126A, C196A, C296A, C331A, or C385A, C418A mutant HIV-1 89.6 *env*. The cells were incubated with C35S Trx (4  $\mu$ M) for 60 min at 37 °C. The reaction was quenched with iodoacetamide, cell lysates prepared, and samples resolved on SDS-PAGE under nonreducing conditions and blotted with anti-Trx (left) or anti-gp120 (right) polyclonal antibodies. Disulfide-linked gp120-Trx complex, unprocessed gp160, and gp120 are indicated. The positions of  $M_r$  markers are shown at left. C35S Trx reacted with wild type and C126A, C196A mutant gp120. Both C296A, C331A and C385A, C418A mutant gp160 proteins were not processed to gp120 and did not react with C35S Trx.



**FIGURE 6. Structure of the V3 domain disulfide bond and mechanism of cleavage.** *A*, ribbon structure of gp120 showing the V3 domain loop (red) and the Cys<sup>296</sup>-Cys<sup>331</sup> disulfide bond (yellow) in space-filling representation. The structure is that of Protein Data Bank code 1YYM (37). A close-up of the Cys<sup>296</sup>-Cys<sup>331</sup> gp120-RHStaple disulfide bond, which constrains a  $\beta$ -loop motif, is shown at right. *B*, mechanism of disulfide bond cleavage. *I*, deprotonation of Cys<sup>331</sup> (blue) of reduced Trx by buffer to give *I* as the initial state. *II*,  $S_N2$  reaction of *I* with the gp120 Cys<sup>296</sup>-Cys<sup>331</sup> disulfide bond (RSSR') with TrxCys<sup>331</sup> nucleophile (blue), gp120Cys<sup>296</sup> electrophile (red), and gp120Cys<sup>331</sup> leaving group (brown) gives *III* via transition state *II* with byproduct gp120Cys<sup>331</sup> (R'SH). *III*, deprotonation of *III* by buffer to give *IV*. *IV*, intramolecular  $S_N2$  reaction of *IV* gives oxidized Trx (*VI*) via transition state *V* with byproduct gp120Cys<sup>296</sup> (RSH). The transition states *II* and *V* are presumed to have the central sulfur atom in a trigonal bipyramidal geometry (when the two nonbonding electron pairs on sulfur are considered as shown) with the S...S...S angle close to or exactly 180°. *C*, overlay ribbon structures of the reduced (green) and oxidized (blue) forms of AtFKBP13 showing the Cys<sup>106</sup>-Cys<sup>111</sup>-RHStaple bond in an extended  $\beta$ -loop structure. The loop shifts position when the bond is reduced. The structures are that of Protein Data Bank codes 1Y00 (oxidized) and 1U79 (reduced) (36).

in V3, often in conjunction with sequence changes in V1/V2 and C4 (20, 28).

The gp120 disulfide bond cleaved by thioredoxin is that which constrains the V3 loop of gp120, Cys<sup>296</sup>-Cys<sup>331</sup> (Fig. 6A). The finding of the unpaired cysteine thiol at Cys<sup>331</sup> in the

C35S Trx-gp120 complex informs the mechanism of cleavage. The Cys<sup>331</sup> sulfur ion nucleophile of thioredoxin attacks the Cys<sup>296</sup> sulfur ion electrophile of the Cys<sup>296</sup>-Cys<sup>331</sup> disulfide bond, cleaving the bond. The TrxCys<sup>331</sup>-Cys<sup>296</sup> gp120 mixed disulfide then spontaneously decomposes via an intramolecu-



lar thiol-disulfide exchange releasing oxidized thioredoxin (Fig. 6B). Interestingly, a recombinant gp140 trimer was found to be disulfide-bonded through the V3 domain (29), which supports the redox activity of the V3 bond.

Binding of purified sCD4 to isolated gp120 or cell surface CD4 to cell surface gp120 enhanced cleavage of the V3 disulfide bond. This result implies that CD4 binding induces a conformational change in gp120 that facilitates reduction of the disulfide bond. This conclusion is supported by the studies of Liu *et al.* (24), who investigated the three-dimensional structures of trimeric env displayed on native HIV-1 in the unliganded state and in complex with CD4. They showed that CD4 binding results in a major reorganization of the env trimer, causing an outward rotation and displacement of each gp120 monomer. This was coupled with a rearrangement of the gp41 trimer, leading to closer contact between the viral and target cell membranes.

We propose the following sequence of events for the contribution of the redox change in gp120 to the mechanism of HIV entry. Binding of HIV gp120 to immune cell CD4 both brings gp120 close to a cell surface oxidoreductase, which could be protein disulfide isomerase (3, 4), thioredoxin (8), or glutaredoxin-1 (13), and induces a conformational change in gp120 that facilitates reduction of the V3 domain disulfide bond by the oxidoreductase. Cleavage of the V3 disulfide bond then results in a conformational change in this domain, which triggers dissociation of chemokine receptor from gp120 and allows refolding of gp41 and viral-membrane fusion.

The observations of Barbouche *et al.* (3) are noteworthy in light of this model. These investigators incubated purified gp120 with the chemical reductant,  $\beta$ -mercaptoethanol, and then measured binding of the reduced gp120 to CD4 and also indirectly to CXCR4 by competition for binding by the CXCR4 ligand, SDF1- $\alpha$ . They found that gp120 containing two unpaired cysteine thiols (one cleaved disulfide bond) bound to CD4 but was less effective than native gp120 at competing for binding of SDF1- $\alpha$  to CXCR4. This finding implies that cleavage of the gp120 disulfide bond reduces the affinity of the protein for CXCR4, but not for CD4. It cannot be assumed that the V3 disulfide bond was the one cleaved by  $\beta$ -mercaptoethanol, however, so this conclusion must be treated with caution with respect to the findings reported herein. For example, the CD4 Cys<sup>130</sup>-Cys<sup>159</sup> disulfide bond is cleaved by thioredoxin, but not by the chemical reductant, dithiothreitol (7).

The V3 disulfide bond has all the hallmarks of an allosteric bond and, notably, has the same configuration as the disulfide bond cleaved in CD4. Allosteric disulfides are defined as bonds that control the function of the mature protein when they are reduced or oxidized (30, 31). A feature of the allosteric disulfides identified so far is that they link  $\beta$ -strands or  $\beta$ -loops (32). They have also been associated with a particular disulfide bond configuration known as the -RHStaple, which reflects the sign of the five  $\chi$  (chi) angles that make up the disulfide bond (30). There are 20 possible disulfide bond configurations based on this measure. A particular property of -RHStaple bonds is the close proximity of the  $\alpha$ -carbon atoms of the two cysteine residues (30).

The Cys<sup>296</sup>-Cys<sup>331</sup> V3 disulfide bond is an -RHStaple in all eight crystal structures of gp120 in the Protein Data Bank (31). It constrains a  $\beta$ -loop motif (Fig. 6B) and has a very short  $\alpha$ -carbon- $\alpha$ -carbon distance of  $3.84 \pm 0.04$  Å (mean  $\pm$  S.E. for the eight structures), compared with a mean of 5.62 Å for all disulfides in a nonredundant set of x-ray structures (31) (Fig. 6A). The disulfide bond cleaved in CD4, Cys<sup>130</sup>-Cys<sup>159</sup> in domain 2, is also an -RHStaple that links adjacent  $\beta$ -strands and has a short  $\alpha$ -carbon- $\alpha$ -carbon distance of 3.3 Å (7, 33).

The fact that the functional disulfides in both CD4 and gp120 have the same unusual configuration likely reflects some common requirement for their reduction. Disulfide bond reduction occurs via a bimolecular nucleophilic substitution ( $S_N2$ ) reaction mechanism (34, 35). This reaction is highly directional and proceeds via a transition state in which the three sulfur atoms involved, in this case the Cys<sup>32</sup> sulfur ion nucleophile of thioredoxin and the two sulfur atoms of the gp120 or CD4 disulfide bond, must form a  $\sim 180^\circ$  angle (Fig. 6B). Steric factors that prevent proper positioning of the three sulfur atoms will preclude any reaction. It may be that the -RHStaple configuration provides for the correct positioning of the three sulfur atoms involved.

The nature of the conformational change in the V3 domain upon reduction of the disulfide bond is not known but may be similar to that observed for cleavage of an analogous -RHStaple bond in the plant peptidyl-prolyl *cis-trans* isomerase, AtFKBP13. Photosynthesis in plants and eukaryotic algae occurs in the chloroplasts and the lumen of these organelles, the thylakoids, is the site of oxygen evolution. There are more than a dozen resident peptidyl-prolyl *cis-trans* isomerases in the thylakoid lumen, one of which is AtFKBP13. The enzyme contains an -RHStaple disulfide bond (Cys<sup>106</sup>-Cys<sup>111</sup>) that is reduced by chloroplast *m*-type thioredoxin (36). Cleavage of the bond results in loss of peptidyl-prolyl isomerase activity. The disulfide links a  $\beta$ -loop motif that shifts position considerably when the bond is reduced (Fig. 6C).

Strategies to prevent cleavage of the gp120 V3 disulfide bond will inhibit HIV infection. This has been achieved *in vitro* by inactivating the oxidoreductase(s) responsible for the cleavage of the V3 bond (3–6, 8, 13). Another means of intervention might be dithiol cross-linking agents that react with the unpaired Cys<sup>296</sup> and Cys<sup>331</sup> thiols and prevent the conformational change in the V3 domain.

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## **Disulfide Bond That Constrains the HIV-1 gp120 V3 Domain Is Cleaved by Thioredoxin**

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