

Disulfide exchange in domain 2 of CD4 is required for entry of HIV-1

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CD4, a member of the immunoglobulin superfamily of receptors that mediates cell-cell interactions in the immune system, is the primary receptor for HIV-1. The extracellular portion of CD4 is a concatenation of four immunoglobulin-like domains, D1 to D4. The D1, D2 and D4 domains each contain a disulfide bond. We show here that the D2 disulfide bond is redox-active. The redox state of the thiols (disulfide versus dithiol) appeared to be regulated by thioredoxin, which is secreted by CD4⁺ T cells. Locking the CD4 and the thioredoxin active-site dithiols in the reduced state with a hydrophilic trivalent arsenical blocked entry of HIV-1 into susceptible cells. These findings indicate that redox changes in CD4 D2 are important for HIV-1 entry and represent a new target for HIV-1 entry inhibitors.

CD4 is a member of the immunoglobulin (Ig) superfamily that binds to class II major histocompatibility complex (MHC) to enhance T cell responses¹. Another ligand for CD4 is the HIV-1 surface protein gp120. HIV-1 binds *via* gp120 to CD4 and the chemokine coreceptor, CCR5 or CXCR4, to trigger fusion of the virus with the cell membrane and mediate entry of HIV-1 into CD4⁺ T lymphocytes and monocyte-macrophages². CD4 has a molecular mass of 55 kD and consists of an extracellular portion (residues 1–371), a transmembrane segment (372–395) and a cytoplasmic tail (396–433)³. The extracellular portion consists of four Ig-like domains^{4,7}, D1 to D4. Class II MHC binding extends over D1 and D2¹, whereas HIV-1 gp120 binds to D1².

The backbone of Ig domains are defined by β strands indicated by letters A through G. Following standard Ig nomenclature, one β sheet of the sandwich-like structure contains strands A, B and E, and the other sheet has strands C, C', F and G. The disulfide bond in D1 of CD4 is between Cys¹⁶ in strand B and Cys⁸⁴ in strand F^{4,6}. Similarly, the disulfide bond in D4 is between Cys³⁰³ in strand B and Cys³⁴⁵ in strand F^{4,7}. In contrast, D2 has a truncated β barrel (75 residues compared to ~100 residues) and a nonstandard disulfide bond between Cys¹³⁰ in strand C and Cys¹⁵⁹ in strand F^{4,6}. In effect, D2 has lost the cysteine in strand B and acquired one in strand C. This means that the disulfide bond in D2 is between strands in the same sheet rather than between sheets, as is more normal. The cysteine in strand C eliminates the normally conserved tryptophan in that position^{4,6}.

The geometry and strain of the D2 disulfide bond is also unusual. The disulfide is right- rather than left-handed and has a short (3.92 Å) C α -C α distance compared to standard Ig disulfides⁸ (6.6 Å) and right-handed disulfides⁸ (5.07 Å). Calculation of the dihedral strain energies^{8,9} of the disulfide bonds reveals that the D2 bond has a high strain energy (4.74 kcal mol⁻¹) compared to the D1 (2.28 kcal mol⁻¹) and D4

(1.71 kcal mol⁻¹) disulfide bonds. A high dihedral strain energy correlates with ease of reduction of the disulfide bond⁸. In addition, the D2 disulfide makes the least contribution to overall stability, as determined by enthalpy calculations¹⁰: D2 disulfide is 3.65 kcal mol⁻¹ compared to the D1 (4.36 kcal mol⁻¹) and D4 (3.97 kcal mol⁻¹) disulfides.

These features led us to investigate whether the D2 disulfide bond of CD4 was redox-active on the cell surface. We show here that the D2 disulfide bond can exist in the reduced dithiol form on the cell surface. Thioredoxin, a small protein reductant that is secreted by CD4⁺ T cells, appeared to regulate the redox state of the D2 disulfide. Reaction of the D2 and thioredoxin active-site dithiols with a peptide trivalent arsenical blocked entry of HIV-1 into susceptible cells. These findings indicate that reduction of the D2 disulfide bond played a role in HIV-1 entry. Perturbation of this redox event may be a worthwhile strategy for inhibiting HIV-1 infection.

Results

Cell-surface CD4 contains free thiol(s)

We labeled CD4⁺ cells with either sulfosuccinimidobiotin (SSB) or 3-(*N*-maleimidylpropionyl)biocytin (MPB). Both reagents are substantially membrane-impermeable^{11,12}. SSB labels primary amines, whereas MPB will only label free thiols. Because there are no unpaired cysteines in the extracellular part of CD4, incorporation of MPB would indicate reduction of one or more of the three disulfide bonds. The thiol specificity of MPB was confirmed by labeling purified plasma proteins that lack or contain a free thiol. MPB labeled serum albumin, which contains a free thiol, but not prothrombin, which does not contain free thiols (data not shown).

Cell-surface CD4 incorporated both SSB and MPB (**Fig. 1a,b**). Labeling with MPB was thiol-specific, as preblocking of the MPB with

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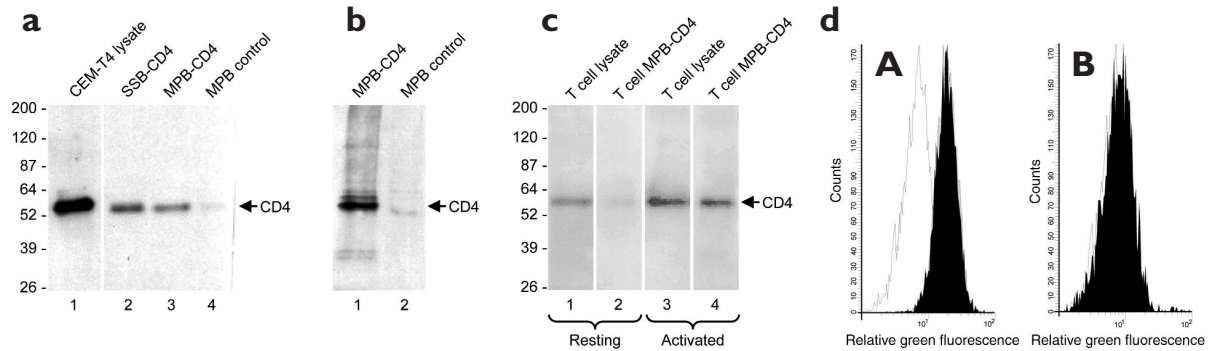


Figure 1. Cell-surface CD4 contains free thiol(s). (a) CEM-T4 cells were labeled with either SSB or MPB; the biotin-labeled proteins were collected and immunoblotted for CD4. Lane 1, CEM-T4 lysate (from 10^6 cells); lane 2, SSB-labeled CEM-T4 CD4; lane 3, MPB-labeled CEM-T4 CD4. Biotin-labeled proteins were from 5×10^6 cells. Lane 4, a control experiment in which MPB was preblocked with GSH before incubation with CEM-T4 cells. The positions of size markers, in kD, are shown. (b) CEM-T4 cells were labeled with MPB; CD4 was immunoprecipitated and blotted with peroxidase-streptavidin. Lane 1, MPB-labeled CD4 (from 7×10^6 cells). Lane 2, a control experiment as in a. (c) Peripheral blood T cells or T cells activated with phytohemagglutinin for 3 days were labeled with MPB; the biotin-labeled proteins were collected and immunoblotted for CD4. Lanes 1 and 3, unactivated and activated T cell lysate (from 10^6 cells), respectively. Lanes 2 and 4, MPB-labeled CD4 from unactivated and activated T cells (from 10^7 cells), respectively. (d) gp120 bound equivalently to unlabeled or MPB-labeled cell-surface CD4. CEM-T4 cells were incubated without (filled histogram) or with (shaded histogram) MPB (A) or Leu3a mAb (B, filled histogram). FITC-gp120 was then added to the cells, and bound gp120 was measured by flow cytometry. The filled and shaded histograms in A overlap. The open histograms in A and B are controls, which used an irrelevant FITC-conjugated mAb.

reduced glutathione (GSH) ablated labeling. Comparison of the amount of SSB- versus MPB-labeled CD4 indicated that ~40% of CD4 on the surface of CEM-T4 cells contained one or more free thiols (Fig. 1c). A substantial fraction, therefore, of one or more of the three CD4 disulfide bonds could exist in the reduced form on the cell surface.

CD4 on the surface of human blood T cells (Fig. 1c), monocyte-macrophages (data not shown) and the human monocyte-macrophage line THP-1 (data not shown) also incorporated MPB. Activation of blood T cells with phytohemagglutinin for 3 days caused a ~45% increase in the fraction of total cell-surface CD4 that labeled with MPB (Fig. 1c). Another Ig fold receptor, Thy-1, was not labeled with MPB on the TIB-47 cell line (data not shown). These results indicated that CD4 on the surface of both T cells and macrophages was redox-active and that cell activation increased the reduction of CD4.

The effect of cell-surface CD4 labeling with MPB on HIV-1 gp120 binding to CD4 was measured by flow cytometry. CEM-T4 cells were labeled with MPB and then incubated with fluorescein isothiocyanate (FITC)-conjugated gp120. Treatment of the T cells with MPB had no effect on binding of gp120 (Fig. 1dA). gp120 bound specifically to cell-surface CD4, as preblocking CD4 with the monoclonal antibody (mAb) Leu3a ablated binding (Fig. 1dB). Reduction of CD4, therefore, did not significantly affect binding of gp120 to D1 of CD4.

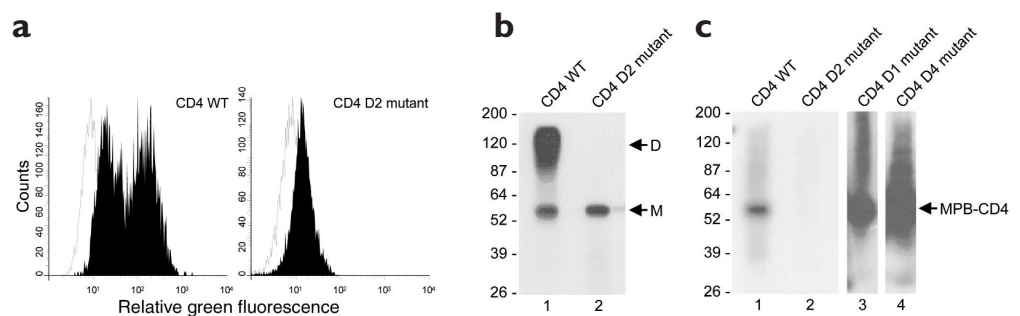
The D2 disulfide of CD4 is redox-active

These labeling experiments showed that one or more of the three disulfide bonds in cell-surface CD4 could exist in the reduced dithiol form. To determine which of the disulfide bonds was redox-active, we stably transfected disulfide bond mutants of CD4 into cultured cells and tested for the presence of free thiols by labeling with MPB. We prepared cysteine→alanine mutants of the three pairs of cysteine residues in D1, D2 or D4 of CD4. Several attempts at stably transfecting the human CD4-T cell line A2.01 with the CD4 disulfide bond mutants in the eukaryotic expression vector SR α ¹³ were unsuccessful (data not shown). Stable transfection of the equivalent cysteine→serine mutants into A2.01 cells was also unsuccessful (data not shown). In contrast, wild-type CD4 was expressed on the surface of A2.01 cells (data not shown). These findings suggested that the disulfide bond mutants were recognized by the T cells as misfolded and targeted for degradation. Transfection and surface expression of the CD4 disulfide bond mutants could be achieved, however, in human fibrosarcoma cells.

Surface expression of wild-type CD4 and the D2 disulfide mutant in HT1080 cells is shown (Fig. 2a). Immunoblotting of the wild-type cell lysate indicated the presence of both monomers and dimers of CD4 (Fig. 2b). The CD4 dimers resolved into monomers upon reduction and alkylation of the lysate, which indicated that the dimers were

Figure 2. The CD4 D2 disulfide is redox-active.

(a) Human fibrosarcoma cells expressing either wild-type (WT) CD4, or a mutant CD4 in which both D2 cysteines are mutated to alanines, were incubated with a control FITC-conjugated mAb (open histograms) or with FITC-conjugated Leu3a mAb (filled histograms); the bound antibodies were measured by flow cytometry. (b) The HT1080 CD4 WT (lane 1) and CD4 D2 mutant (lane 2) cells were lysed, and lysate from 2×10^5 cells was immunoblotted for CD4. M, CD4 monomer; D, CD4 dimer. (c) The HT1080 CD4 WT (lane 1) and D2 (lane 2), D1 (lane 3) and D4 (lane 4) mutant cells were labeled with MPB and the biotin-labeled proteins collected and immunoblotted for CD4. MPB-labeled proteins were from 7×10^6 cells. The positions of size markers, in kD, are shown.



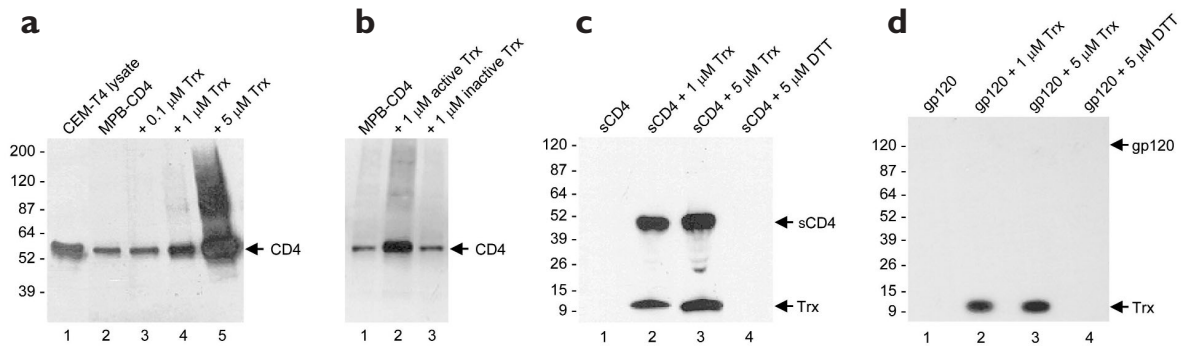


Figure 3. Reduction of cell-surface and soluble CD4 by thioredoxin. (a) CEM-T4 cells were incubated without (lane 2) or with increasing concentrations of human thioredoxin (Trx) (lanes 3–5), labeled with MPB and the biotin-labeled proteins collected and immunoblotted for CD4. Lane 1, CEM-T4 lysate. The positions of size markers, in kD, are shown. (b) CEM-T4 cells were incubated with either redox-active thioredoxin mutant (lane 2) or redox-inactive thioredoxin mutant (lane 3), labeled with MPB and the biotin-labeled proteins were collected and immunoblotted for CD4. Lane 1, untreated CEM-T4 cells. (c) sCD4 was incubated without (lane 1) or with (lanes 2 and 3) thioredoxin or dithiothreitol (lane 4) and then labeled with MPB and blotted with peroxidase-streptavidin. MPB-labeled sCD4 and thioredoxin are shown. (d) gp120 was incubated without (lane 1) or with (lanes 2 and 3) thioredoxin or dithiothreitol (lane 4), then labeled with MPB and blotted with peroxidase-streptavidin. MPB-labeled thioredoxin is shown.

disulfide-bonded (data not shown). The extent of dimer formation varied with each experiment, but was always apparent. CD4 D1 and D4 disulfide mutants also formed dimers (data not shown). In contrast, the CD4 D2 disulfide mutant did not form dimers (Fig. 2b). Labeling of wild-type and the disulfide mutant CD4s on the surface of HT1080 cells with MPB is shown (Fig. 2c). The monomeric forms of wild-type CD4 and the D1 and D4 disulfide bond mutants incorporated MPB, whereas the D2 disulfide mutant was not labeled, despite similar cell-surface expression of the monomeric forms of the wild-type and mutant CD4s (data not shown). These results indicated that the D2 disulfide of CD4 could exist in the reduced dithiol form on the cell surface and that it was involved in thiol-dependent dimerization of CD4.

Reduction of CD4 by thioredoxin

The finding that blood T cell activation resulted in reduction of the D2 disulfide in cell-surface CD4 suggested that the oxidation state of the D2 disulfide bond was controlled by the cell (Fig. 1c). This may be accomplished by the secretion of a disulfide bond reductase by the CD4⁺ T cell. Incubation of CEM-T4 cells with increasing concentrations of the protein reductant thioredoxin resulted in increasing disulfide reduction of cell-surface CD4 (Fig. 3a). The redox properties of thioredoxin were required for reduction of cell-surface CD4, as a redox-inactive thioredoxin mutant¹⁴ did not reduce CD4 (Fig. 3b). Incubation of CEM-T4 cells with the same concentrations of another disulfide bond reductase, protein disulfide isomerase, also failed to increase labeling of CD4 with MPB (data not shown).

Soluble CD4 containing the four Ig-like domains (sCD4)¹⁵ was tested for disulfide reduction by thioredoxin. Thioredoxin-facilitated

reduction of sCD4 and incorporation of MPB is shown (Fig. 3c). Disulfide reduction was selective for thioredoxin because equivalent concentrations of the strong reductant dithiothreitol (Fig. 3c) or protein disulfide isomerase (data not shown) did not reduce sCD4. Soluble gp120 could not be reduced by thioredoxin or dithiothreitol (Fig. 3d). Reduction of the CD4 D2 disulfide, therefore, could occur independent of the cell surface and is selective for thioredoxin.

Labeling of CD4 with trivalent arsenic

The proximity of the two free thiols generated in D2 by reduction of the disulfide bond was examined with the chemistry of trivalent arsenic. Trivalent arsenicals form high-affinity ring structures with closely spaced dithiols, but react very poorly with monothioles or dithiols that are not in close proximity^{16–18}. We attached the trivalent arsenical phenylarsenoxide to the thiol of reduced glutathione to produce 4-(*N*-(*S*-glutathionylacetyl)amino)phenylarsenoxide (GSAO)¹⁸. GSAO is substantially membrane-impermeable and binds tightly to synthetic and peptide dithiols and the thioredoxin active-site dithiol, but not to monothioles¹⁸. To detect incorporation of GSAO into CD4, a biotin moiety was attached through a spacer arm to the primary amino group of the γ -glutamyl residue of GSAO (GSAO-B)¹⁸. Cell-surface CD4 incorporated GSAO-B (Fig. 4). The labeling of CD4 with GSAO-B was specific, as a fourfold molar excess of the small synthetic dithiol dimer-captopropanol competed with GSAO-B for labeling. As the CD4 D2 thiols were sufficiently close to complex with a trivalent arsenical, they were predicted to be close enough to reoxidize to the disulfide bond. This result indicated that reduction of the D2 disulfide bond was likely to be reversible.

Inhibition of HIV-1 entry by GSAO

The requirement for redox changes in CD4 for HIV-1 entry into susceptible cells was evaluated by measuring viral DNA accumulation after a high multiplicity HIV-1_{HXB2} infection of HuT-78 T cells in the presence of GSAO¹⁹. Cells were preincubated with GSAO or the corresponding pentavalent arsenical 4-(*N*-(*S*-glutathionylacetyl)amino)phenylarsonic acid (GSAA)¹⁸, before infection with HIV-1_{HXB2}. GSAA controls for the dithiol-reactivity of GSAO, as pentavalent arsenicals do not react with thiols. The reverse transcriptase inhibitor lamivudine (3TC), which inhibits HIV-1 DNA synthesis after viral entry, was used as a positive control. Infected cells were washed and then treated with trypsin to minimize any cell surface-associated virus and then cultured

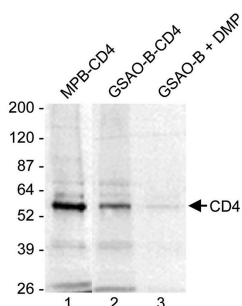
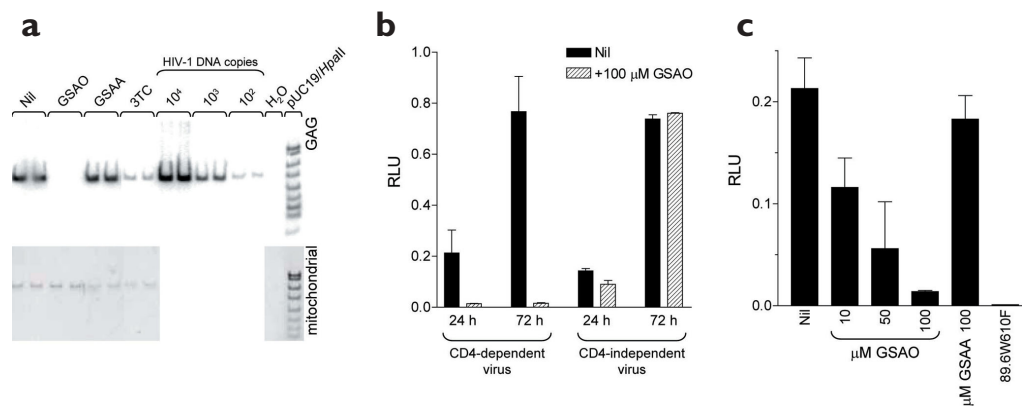


Figure 4. Labeling of cell-surface CD4 with GSAO-B. CEM-T4 were labeled with either MPB (lane 1) or GSAO-B (lane 2); the biotin-labeled proteins were collected and immunoblotted for CD4. Lane 3, a control experiment in which GSAO-B was incubated with CEM-T4 cells in the presence of a fourfold molar excess of 2,3-dimercaptopropanol. Biotin-labeled proteins were from 2×10^6 cells. The positions of size markers, in kD, are shown.

Figure 5. Inhibition of HIV-1 entry into CD4⁺ cells by GSAO. (a) Effect of 100 μ M GSAO or GSAA, or 10 μ M of the reverse transcriptase inhibitor 3TC, on the accumulation of HIV-1 DNA 8 h after infection of HuT-78 cells with HIV-1_{HXB2}. Quantitative PCR amplification of *gag* DNA sequences (upper panel) was done on cell-equivalent amounts of extrachromosomal DNA from duplicate cultures. The HIV-1 DNA standards used were known cell-equivalents of chromosomal DNA isolated from a mixture of three persistently infected cell lines. (b) Effect of GSAO on entry of a CD4-dependent or -independent reporter virus into CD4⁺ or CD4⁻ cells. Pseudotyped NL4-3-Luc.R-E⁻ virus containing *Env* that confers dual tropism (89.6) or a mutant *Env* that uses CXCR4 for entry and is unable to bind CD4 (8x3D368R) were incubated with CD4⁺ (A3.01) or CD4⁻ (CXCR4⁺293T) cells, in the absence or presence of 100 μ M GSAO for 1 h. Cells were cultured in the absence or presence of 100 μ M GSAO for 1 h. Cells were cultured for 24 h or 72 h and analyzed for luciferase expression. Data are mean \pm range from duplicate experiments. (c) Concentration dependence of GSAO. Pseudotyped NL4-3-Luc.R-E⁻ virus containing a dual-tropic *Env* (89.6) or a fusion defective *Env* mutant (89.6W610F) was incubated with CD4⁺ A3.01 T cells in the absence or presence of 10–100 μ M GSAO or GSAA for 1 h. Cells were cultured in the absence or presence of 10–100 μ M GSAO or GSAA for 24 h and analyzed for luciferase expression. Data are mean \pm range of duplicate experiments.



for 8 h. Viral *gag* DNA was then detected by quantitative polymerase chain reaction (PCR). Reverse-transcribed viral DNA was evident in Nil- (as in untreated) and GSAA-treated cultures, but was absent or substantially reduced in cultures treated with GSAO or 3TC, respectively (Fig. 5a). Neither GSAO nor GSAA had any effect on cell viability (data not shown). These results show that GSAO inhibited HIV-1 infection of HuT-78 T cells at a stage before reverse transcription of the viral genome, which is consistent with entry-level inhibition.

To test the requirement of CD4 for the inhibitory effects of GSAO on HIV-1 entry, inhibition of CD4-dependent or -independent reporter virus entry into CD4⁺ or CD4⁻ cells by GSAO was measured. GSAO (100 μ M) effectively blocked the entry of a dual-tropic²⁰ (HIV-1_{89.6}) reporter virus into CD4⁺ A3.01 T cells, but had no significant effect on entry of a reporter virus that uses CXCR4 for entry (HIV-1_{8x3D368R})²¹ into CXCR4⁺CD4⁻ 293T cells (Fig. 5b). GSAA (100 μ M) had no effect on either CD4-dependent or -independent virus entry (data not shown). This result also controlled for possible effects of GSAO on events after viral entry and before expression of the viral DNA. The half-maximal inhibitory concentration of GSAO in this system was \sim 10 μ M (Fig. 5c). HIV-1 entry measured by this system was specific, as a fusion defective mutant of HIV-1_{89.6} (89.6W610F) (data not shown) did not enter CD4⁺ cells (Fig. 5c).

These observations indicated that locking the CD4 and the thioredoxin active-site dithiols in the reduced state with a trivalent arsenical blocked entry of HIV-1 into CD4⁺ cells.

Discussion

The CD4 D2 disulfide bond is unusual because it links cysteines within the one β -sheet rather than across a barrel and is right- rather than left-handed. It was not surprising, therefore, that the bond is particularly strained in comparison to the D1 and D4 disulfide bonds. The more strain on a disulfide bond, the more readily it is reduced⁸. These features of the D2 disulfide bond suggest that it could be reduced on the surface of cells. Indirect support for this theory was provided by the finding that thiol-oxidizing reagents such as HgCl₂²² and 5,5'-dithiobis(2-nitrobenzoic acid)²³ facilitate dimerization and oligomerization of CD4 on the cell surface and that uptake of HIV-1 by CD4⁺ T cells is inhibited by membrane-impermeable thiol-reactive reagents²⁴. Using MPB, we showed here that

the D2 disulfide of CD4 is redox-active. HIV-1 entry into T cells was dependent on the redox state of CD4.

Disulfides that bridge across the nearest neighbor positions in antiparallel β -strands are uncommon²⁵, although crystal structures of model cysteine peptides²⁶ and antiparallel dimers²⁷ have demonstrated that disulfide bridging between strands in antiparallel β -sheets can occur. Side-by-side disulfide bonds have been predicted in other members of the Ig superfamily²⁸. The Ig domain of the α chain of CD8, a receptor with a similar function to CD4, also has an unconventional disulfide linkage²⁹. There are three cysteines in this Ig domain: one each in stands B and F, as usual, and a third that is two residues before the conserved tryptophan in strand C. The disulfide in this domain links this extra cysteine in strand C with the cysteine in strand B, leaving the cysteine in stand F unpaired²⁹. The disulfide bond in Ig folds is usually between the cysteines in strands B and F.

Reduction of disulfide bonds is usually facilitated by another redox protein. It is likely, therefore, that reduction of cell-surface CD4 was facilitated by a cell-derived factor. Thioredoxin is a 12-kD redox protein³⁰ that is secreted by CD4⁺ T cells and binds to their surface^{31–34}. Indeed, we showed that thioredoxin reduced cell-surface and soluble CD4, but not soluble gp120. CD4⁺ T cell activation is associated with increased secretion of thioredoxin^{31,33}, which is consistent with the increased reduction of peripheral blood T cell-surface CD4 upon phytohemagglutinin activation. It appears, therefore, that the redox state of cell-surface CD4 is controlled by thioredoxin secreted by the same or other cells.

The question of whether the D2 disulfide can exchange between the oxidized and reduced forms is an important one for CD4 function. Disulfide exchange in D2 is supported by the link between cell activation and reduction of cell-surface CD4. Reoxidation of the dithiol would imply that reduction of the disulfide did not result in significant separation of the thiols. The spacing of the D2 thiols was examined with a hydrophilic trivalent arsenical. Trivalent arsenicals react with thiols that are within \sim 10 Å of each other to form stable cyclic dithioarsinates¹⁶, but react very poorly with monothiols or dithiols that are not in close proximity¹⁸. For instance, the hydrophilic trivalent arsenical GSAO binds tightly to the active site dithiol of thioredoxin but does not bind to the single free thiol of albumin¹⁸. GSAO also bound to

the reduced dithiol form of CD4. This result implied that the two thiols of reduced CD4 were in close proximity, and—by analogy with the dithiol of thioredoxin—are predicted to be close enough to reoxidize to the disulfide bond. Dynamic exchange of the D2 disulfide–dithiol, therefore, may regulate CD4 structure and function.

These findings suggested that the oxidized and reduced forms of CD4 exist on the cell surface in equilibrium and are controlled by thioredoxin secreted by the T cell. We propose that reduction of the D2 disulfide by thioredoxin will result in oxidation of the dithiol active-site of thioredoxin. For thioredoxin to catalyze reduction of another CD4 molecule, a mechanism is required to reduce the oxidized form of the protein. This may be accomplished by thioredoxin reductase, which is secreted by peripheral blood mononuclear cells and is present in plasma at a concentration of 18 ng ml⁻¹ (ref. 35). It is important to note, however, that our findings do not exclude the possibility that thioredoxin-mediated exchange of the D2 disulfide may occur indirectly through redox control of another cell-surface protein.

Perturbation of the redox chemistry of the D2 dithiol and thioredoxin by the hydrophilic trivalent arsenical blocked HIV-1 entry into susceptible cells in a CD4-dependent manner. The process of HIV-1 infection begins with binding of the viral envelope glycoprotein, gp120, to both the D1 domain of CD4 and either CCR5 or CXCR4. Infection is complete once the virus fuses with the cell membrane². The reason why redox activity of the D2 disulfide is important for HIV-1 infection is unknown. The viral envelope protein gp120 bound equivalently to unlabeled or GSAO- or MPB-labeled cell-surface CD4. This finding implied that redox changes in the D2 disulfide bond were not important for initial binding of HIV-1 but rather for post-binding events before fusion.

It is likely that disulfide exchange in D2 is involved in conformational changes in CD4 after binding of HIV-1^{36,37}. For instance, a mAb that recognizes D2 blocks post-binding events relevant to plasma membrane-virus envelope fusion without affecting the interaction of virions with CD4^{38,39}. It is also possible that disulfide-dependent self-association of CD4 through the D2 domain plays a role in HIV-1 entry²³.

Notably, plasma concentrations of thioredoxin in HIV-1-infected individuals are inversely correlated with CD4⁺ cell numbers⁴⁰. In addition, survival of HIV-1-infected individuals with chronically increased plasma thioredoxin concentrations and <200 CD4⁺ cells per μ l is significantly impaired relative to infected individuals with >200 CD4⁺ cells per μ l⁴¹. Also, the thiol-reactive compounds *N*-acetyl-L-cysteine^{42,43}, cystamine^{44,45}, cysteamine⁴⁶ and *D*-penicillamine^{47,48} all have anti-HIV-1 activity. It may be that the efficacy of these drugs are, in part, due to formation of mixed disulfides with the dithiol forms of thioredoxin and/or CD4.

Despite the success in developing regimens that block HIV-1 transcription and assembly, problems of drug resistance, latent viral reservoirs and drug toxicity highlight the need for drugs with different modes of action. In particular, compounds that block HIV-1 entry would complement those that interfere with viral replication^{2,49}. The redox changes in D2 we describe here represent a new target for HIV-1 entry inhibitors. Dithiol-reactive compounds such as GSAO are one way of blocking this redox chemistry.

Methods

Labeling of CD4⁺ cells or sCD4. Peripheral blood mononuclear cells were prepared from fresh citrated blood by Ficoll-Hypaque density gradient separation. Monocytes were separated from the T cells by allowing them to attach to tissue-culture plastic overnight. The unattached T cells were depleted of CD8⁺ cells with antibody-conjugated magnetic microbeads. Blood T cells or CEM-T4 cells (NIH AIDS Research and Reference Reagent Program), 1 ml of 5×10^6 – 10×10^6 cells ml⁻¹ in Hank's balanced salt solution, were incubated with SSB (Pierce, Rockford, IL), MPB (Molecular Probes, Eugene, OR) or GSAO-B (100 μ M) for 30

min at room temperature. On one occasion, MPB was preblocked with reduced glutathione (GSH) before incubation with cells or cells were labeled with GSAO-B in the presence of 2,3-dimercaptopropanol (400 μ M, Fluka, Sydney, Australia). On another occasion, cells were preincubated with thioredoxin or dithiothreitol for 1 h at 37 °C, then labeled with MPB. The thioredoxin active site cysteines, residues 32 and 35, were replaced by serines in the redox-inactive mutant. Another pair of conserved cysteines, residues 69 and 73, were replaced by serines in the redox-active mutant¹⁴. Unreacted SSB was quenched with glycine (200 μ M), whereas unreacted MPB was quenched with GSH (200 μ M) for 30 min at room temperature. The labeled cells were washed three times with PBS and sonicated in 1 ml of ice-cold buffer (50 mM Tris HCl and 0.15 M NaCl at pH 8.0) containing 1% Triton X-100, 10 μ M leupeptin, 10 μ M aprotinin, 2 mM phenylmethylsulfonyl fluoride and 5 mM EDTA. Streptavidin-agarose beads (50 μ l of packed beads, Sigma-Aldrich, Sydney, Australia) were incubated with the cell lysates for 1 h at 4 °C on a rotating wheel to isolate the biotin-labeled proteins. The streptavidin-agarose beads were washed five times with buffer (50 mM Tris HCl and 0.15 M NaCl at pH 8.0) containing 0.05% Triton X-100, and the biotin-labeled proteins were released from the beads by boiling in 30 μ l of SDS-Laemmli buffer for 2 min. Samples were resolved on 4–15% SDS-PAGE under nonreducing conditions and transferred to polyvinylidene difluoride (PVDF) membrane. Proteins were detected by immunoblot with 5 μ g ml⁻¹ of anti-murine CD4 (mAb Leu3a, Becton Dickinson, Bedford, MA) and a 1:2000 dilution of horseradish peroxidase (referred to as peroxidase throughout)—rabbit anti-mouse (Dako, Carpinteria, CA). Chemiluminescence films were analyzed with a GS-700 Imaging Densitometer and Multi-Analyst software (BioRad, Hercules, CA).

sCD4 and HIV-1_{SF2} gp120 were from the NIH AIDS Research and Reference Reagent Program. sCD4 or gp120 (10 μ g ml⁻¹) was incubated without or with thioredoxin or dithiothreitol for 10 min at 37 °C and then labeled with MPB (100 μ M) for 30 min at room temperature. The unreacted MPB was quenched with GSH (200 μ M) for 30 min at room temperature and the sCD4 or gp120 (0.1 μ g) was resolved on SDS-PAGE and blotted with streptavidin peroxidase to detect the biotin label.

Immunoprecipitation of CD4. CEM-T4 cells were labeled with MPB, incubated with Leu3a mAb (5 μ g ml⁻¹) for 30 min, washed three times and lysed in 0.5 ml of buffer (50 mM Tris HCl, 0.15 M NaCl at pH 8.0) containing 0.5% Triton X-100, 0.05% Tween 20, 100 μ g ml⁻¹ bacitracin, 2 mM benzamide and 2 mM phenylmethylsulfonyl fluoride for 30 min at 4 °C. The detergent-insoluble material was removed by centrifugation at 12000g for 30 min, and the supernatant was incubated with 10⁷ goat anti-mouse IgG-coated Dynabeads (Dyna, Melbourne, Australia) for 60 min. All incubations were at 4 °C. The beads were washed and the bound CD4 released by boiling the beads in 30 μ l of SDS-Laemmli buffer for 2 min. Samples were resolved on 4–15% SDS-PAGE under nonreducing conditions and transferred to PVDF membrane. MPB-labeled proteins were blotted with peroxidase-streptavidin (Amersham, Sydney, Australia) and used at a 1:1000 dilution.

gp120 flow cytometry assay. HIV-1_{SF2} gp120 was labeled with FITC (Molecular Probes), according to the manufacturer's instructions. CEM-T4 cells (10⁶ cells ml⁻¹) were incubated without or with MPB (100 μ M) for 30 min at 37 °C in serum-free media. On one occasion, cells were incubated with Leu3a mAb (20 μ g ml⁻¹) for 30 min at 4 °C in serum-free media. FITC-gp120 (10 nM final concentration) was then added to the cells and incubated for 1 h at 37 °C. The cells were washed twice with PBS that contained 2% fetal bovine serum, and binding of gp120 to the cells was measured by flow cytometry with a FACStar Plus cytometer (Becton Dickinson). Ten-thousand cells were acquired at a flow rate of 500–1000 particles per second.

Mutation of CD4. The T4-pMV7 plasmid, which contains full-length human CD4 cDNA, was from the NIH AIDS Research and Reference Reagent Program. The mammalian expression vector pcDNA3, which contains a CMV promoter, was from Invitrogen (San Diego, CA). A 3-kb CD4 cDNA was extracted from the T4-pMV7 plasmid as a *EcoRI* fragment and subcloned into the pcDNA3 vector to produce pcDNA3/CD4 wild-type. The pairs of cysteine residues, which constitute the disulfide bonds in D1 (Cys³⁶ and Cys⁶⁴), D2 (Cys¹³⁰ and Cys¹⁵⁹) and D4 (Cys³⁰³ and Cys³⁴⁵), were individually mutated to alanine or serine residues. The first cysteine in each domain was mutated to alanine or serine with the QuikChange Site-Directed Mutagenesis Kit (Integrated Sciences, La Jolla, CA). The single cysteine mutants were then used as templates to mutate the other cysteine. All mutations were confirmed by automatic sequencing (ABI-377 Automatic Sequencer, Applied Biosystems, Foster City, CA). Human fibrosarcoma HT1080 cells (ATCC, Rockville, MD) (10⁵ cells) were transfected with pcDNA/CD4 vectors (0.5–10 μ g) with Fugene 6 (3–15 μ l, Boehringer, Mannheim, Germany). Stably transfected cells were selected by incubation with medium containing 400 μ g ml⁻¹ of G418 (Life Technologies, Paisley, UK). Surface expression of CD4 was measured by flow cytometry with FITC-conjugated Leu3a mAb and a FACStar Plus cytometer (Becton Dickinson). Ten-thousand cells were acquired at a flow rate of 500–1000 particles per second.

gag PCR assays. HIV-1_{HXB2} infection of the CD4⁺ T lymphoblastoid cell line HuT-78 was done as described¹⁹. Briefly, virus inoculum was obtained by high-density culture of the persistently HTLV-IIIB (HIV-1_{HXB2})-infected H3B cell line⁵⁰ and was determined to have a TCID₅₀ of 3.16×10^6 U ml⁻¹. Subcultured HuT-78 cells were infected at an MOI of 1 with a centrifugal enhancement infection protocol¹⁹. GSAO, GSAA or 3TC were preincubated with cells for 45 min and then washed once in culture media before infection. After centrifugation with virus, cells were washed once in serum-free media, treated with 0.05% trypsin for 5 min at 37 °C to minimize trapped virus and then washed twice in culture medium before plating

in a 48-well tray at a density of 10^6 cells ml^{-1} and culturing for 8 h. Mitochondrial PCRs were done with primers M1 (5'-GACGTTAGGTCAGGTTGAG-3') and M2 (5'-GGTGTCTG GTAGTAAGTG-3') on ~50 cell-equivalents of Hirt supernatant DNA¹⁹. The *gag* PCRs were with primers GAG-P1(+) (5'-GAGGAAGCTGCAGAATGGG-3') and GAG-III(-) (5'-CTGTGAAGCTTGCTCGGCTC-3') on ~1000 cell-equivalents of Hirt supernatant DNA¹⁹. Southern hybridizations were done with [α -³²P]dATP-labeled fragments corresponding to nt 1320–1715 of the human mitochondrial DNA sequence and to nt 1408–1722 of the HIV-1_{HXB2} sequence. The HIV-1 DNA copy number standard used was a mixture of equivalent amounts of chromosomal DNA extracted from known numbers of H3B, ACH-2 and 8E5 persistently infected cells containing two, one and one copies of integrated HIV-1 DNA, respectively¹⁹.

Pseudotyped reporter virus assays. Pseudotyped NL4-3-Luc.R.E. virus stocks were generated by transfecting 293T cells with pNL4-3-Luc.R.E. (NIH AIDS Research and Reference Reagent Program) and a pcDNA3.1 expression plasmid containing HIV-1 *Env* from either HIV-1_{89.6} (dual-tropic)²⁰, HIV-1_{89.6W10F} (a fusion defective mutant of 89.6)²⁰ or HIV-1_{89.6SR} (a mutant variant of HIV-1_{89.6} that uses CXCR4 for entry and is unable to bind CD4)²¹. All transfections were with 1 μg of DNA and Fugene 6 transfection reagent (Roche Diagnostics, Mannheim, Germany). Virus-containing supernatants were collected 72 h after transfection, filtered and frozen at -80°C .

CD4⁺ A3.01 T cells (1×10^6 cells in 1 ml) or 293T cells (0.25×10^6 cells in 1 ml) transfected with pc.FUSIN (NIH AIDS Research and Reference Reagent Program) were preincubated with 10–100 μM GSAO or GSAA for 30 min at 37°C in 12-well plates in serum-free media. Reporter virus (0.5 ml) was then added for 1 h at 37°C . Cells were washed twice in serum-free media and then treated with 0.05% trypsin for 5 min at 37°C to minimize trapped virus¹⁹. Cells were washed another two times in serum-containing media and incubated in 12-well plates in complete media containing 10–100 μM GSAO or GSAA for 24 or 72 h. Luciferase activity was measured using the Promega luciferase assay system (Madison, WI) and expressed as relative light units (RLU).

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Competing interests statement

The authors declare that they have no competing financial interests.

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Disulfide exchange in domain 2 of CD4 is required for entry of HIV-1

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In the AOP version of this article some text was incorrect. On page 3, column 2, line 6 from the bottom the sentence should read “GSAA controls for the dithiol-reactivity of GSAO, as pentavalent arsenicals do not react with thiols.” On page 6, column 1, line 5, a closing parenthical mark, rather than the second prime mark, should appear at the end of the GAG-III (–) sequence. In Figure 3a and b, the arrows to the right of the immunoblot panels, which show the position of CD4, should be labeled “CD4”, not “sCD4”. These errors have been corrected in the HTML version and will appear correctly in print. The PDF version available online has been appended.