Reduced Monomeric CD4 Is the Preferred Receptor for HIV*

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CD4 is a co-receptor for binding of T cells to antigen-presenting cells and the primary receptor for the human immunodeficiency virus type 1 (HIV). CD4 exists in three different forms on the cell surface defined by the state of the domain 2 cysteine residues: an oxidized monomer, a reduced monomer, and a covalent dimer linked through the domain 2 cysteines. The disulfide-linked dimer is the preferred immune co-receptor. The form of CD4 that is preferred by HIV was examined in this study. HIV entry and envelope-mediated cell-cell fusion were tested using cells expressing comparable levels of wild-type or disulfide bond mutant CD4 in which the domain 2 cysteines were mutated to alanine. Eliminating the domain 2 disulfide bond increased entry of HIV reporter viruses and enhanced HIV envelope-mediated cell-cell fusion 2–4-fold. These observations suggest that HIV enters susceptible cells preferably through monomeric reduced CD4, whereas dimeric CD4 is the preferred receptor for binding to antigen-presenting cells. Cleavage of the domain 2 disulfide bond is possibly involved in the conformational change in CD4 associated with fusion of the HIV and cell membranes.

CD4 is a type I integral membrane glycoprotein that plays a central role in the immune response by functioning as a co-receptor for binding of T cell receptor to antigen-class II major histocompatibility complex (MHCII) on antigen-presenting cells (1). It helps shape the T cell repertoire during development of the thymus and is critical for appropriate activation of peripheral T cells. CD4 interacts with nonpolymorphic regions of MHCII on antigen-presenting cells, which increases the half-life of association between the cells (2). The cytoplasmic tail interacts with p56ck protein-tyrosine kinase of the Src family to mediate signal transduction (3).

CD4 is also the primary receptor for the human immunodeficiency virus type 1 (HIV). The HIV envelope glycoprotein (env) 7 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 (4). env is activated by binding to CD4 and chemokine receptor CXCR4 (X4) or CCR5 (R5) on susceptible cells. gp120 dissociates 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 (5).

CD4 consists of one V-type (domain 1) and three C2-type (domains 2, 3, and 4) immunoglobulin-like domains, a transmembrane domain, and a short cytoplasmic region. Immunoglobulin domains are defined by two β sheets that oppose each other in what has been called a barrel or sandwich-like structure. Each β sheet contains seven β strands and usually a single disulfide bond that links across the sheets. Domain 2 of CD4 is most unusual in that the disulfide bond, Cys130–Cys159, links adjacent strands in the same β sheet and has a –RHStaple configuration. We have called this type of bond a cross-strand or allosteric disulfide (6–8). The domain 2 –RHStaple disulfide is reduced by the oxidoreductase, thioredoxin, that is secreted by CD4 + cells (9, 10).

Cleavage of the domain 2 disulfide on the cell surface leads to the formation of covalent dimers of CD4 that are linked through the domain 2 cysteines (9, 11). As the two cysteine residues are partially buried in the crystal structure a conformational change in the domain is predicted to take place for the disulfide-linked dimer to form. Swapping of the domain with formation of two disulfide bonds between Cys130 in one monomer and Cys159 in the other is the likely mechanism of dimer formation (12, 13). The functional significance of dimer formation for immune co-receptor function was tested using cells expressing wild-type or disulfide bond mutant CD4, in which both domain 2 cysteines were mutated to alanine. Eliminating the D2 disulfide bond markedly impairs CD4 immune function, implying that disulfide-linked dimeric CD4 is the preferred form for binding to antigen-presenting cells (13).

The question we have asked in this study is what form of CD4, oxidized or reduced monomer or disulfide-linked dimer, does HIV employ to infect cells? Our findings imply that HIV has a preference for reduced monomeric CD4.

EXPERIMENTAL PROCEDURES

Determination of the Redox Potential of the CD4 Cys130–Cys159 Disulfide Bond—Recombinant human CD4 domain 2 (residues 125–202; ProSpec-Tany TechnoGene Ltd., Rehovot, Israel) was incubated at 7 μM concentration with 0.1 M sodium phosphate, pH 7.0, buffer containing 0.1 mM EDTA, 75 mM 4,5-dihydroxy-1,2-dithiane (DTTred; Sigma) and varying concentrations of 1,4-dithiothreitol (DTTox; Sigma) for 15 h at 30 °C. The redox state of the Cys130–Cys159 bond was mea-
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...sured from differential staining by colloidal Coomassie of the protein resolved on NuPAGE Novex 4–12% BisTris gel. The data were expressed as ratio of reduced to oxidized protein and fit to Equation 1,

\[
R = \frac{[\text{DTT}^{\text{red}}]/[\text{DTT}^{\text{ox}}])}{K_{eq} + [\text{DTT}^{\text{red}}]/[\text{DTT}^{\text{ox}}])}
\]

where \( R \) is the fraction of reduced protein at equilibrium and \( K_{eq} \) is the equilibrium constant. The standard redox potential (\( E^{\circ} \)) of the Cys\(^{130}\)-Cys\(^{159}\) bond was calculated via the Nernst equation,

\[
E^{\circ} = E^{\circ}_{\text{DTT}} - (RT/2F)\ln K_{eq}
\]

using a value of –307 mV for the redox potential of the DTT disulfide bond (14).

**Mechanism-based Kinetic Trapping of the CD4 Cys\(^{130}\)-Cys\(^{159}\) Disulfide with Human Thioredoxin 1**—Full-length wild-type human thioredoxin 1 or the C35S mutant cDNAs in the *Escherichia coli* pQE-60 expression vector were kindly provided by Dr. Tobias Dick, German Cancer Research Center, Heidelberg, Germany. The recombinant proteins were produced as described (15, 16). CD4 domain 2 (7.0 kDa) was incubated with wild-type thioredoxin or C35S trapping mutant (2.6 kDa) in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1 mM EDTA for 1 h at 25 °C. The proteins were resolved on NuPAGE Novex 4–12% BisTris gel under nonreducing and reducing conditions and stained with colloidal Coomassie.

**Construction of Wild-type and C130A,C159A Mutant CD4 Expression Vectors**—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. The mammalian expression vector pCDNA3 which contains a CMV promoter was from Invitrogen. A 3-kb CD4 cDNA was extracted from the T4-pMV7 plasmid as an EcoRI fragment and subcloned into the vector to produce pCDNA3/wt CD4. The pair of cysteine residues which constitute the disulfide bond in domain 2 (Cys\(^{130}\) and Cys\(^{159}\)) were individually mutated to alanine using a QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) to produce pCDNA3/C130A,C159A CD4. A 3-kb CD4 wild-type or mutant cDNA was extracted from the pCDNA3 vectors as an EcoRI fragment and subcloned into the pTRE Tight vector (Clontech) to produce pTRE Tight/wt CD4 and pTRE Tight/C130A,C159A CD4. All primers were purchased from Proligo Primers and Probes (Lismore, NSW).

**Cell Surface Expression of CD4**—The Tet-On Advanced Inducible Gene Expression System (Clontech) was used according to the manufacturer’s instructions. The inducible human embryonic kidney cell line, HEK 293 Tet-On Advanced Cell Line (HEK Tet-On), was cultured in DMEM, pH 7.2, containing 10% Tet fetal calf serum with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mm L-glutamine, and 2.2 gliter\(^{-1}\) sodium bicarbonate at 37 °C and 5% CO\(_2\). The HEK Tet-On cells were transfected with pTRE Tight/wt CD4 and pTRE Tight/C130A,C159A CD4 vectors and the hygromycin linear marker using FuGENE 6 (Roche Applied Science). Stably transfected cells were selected by incubation with medium containing 50 μg/ml hygromycin and 100 μg/ml G418 (Invitrogen). Hygromycin-resistant colonies were selected and tested for doxycycline-regulated CD4 gene expression.

**Detection of Cellular and Surface CD4**—HEK Tet-On cell lysate was prepared at 4 °C in 0.5 ml of 50 mM Tris buffer, pH 8.0, containing 0.5 M NaCl, 1% Triton X-100, 10 μM leupeptin, 10 μM aprotinin, 2 mM phenylmethanesulfon fluoride, and 5 mM EDTA, and samples were resolved on NuPAGE Novex 4–12% BisTris gel with MOPS running buffer under nonreducing conditions and transferred to polyvinylidene difluoride membrane. Proteins were detected by Western blotting using 5 μg/ml anti-CD4 murine monoclonal antibody Leu3a (Becton Dickinson) and a 1:2000 dilution of rabbit anti-mouse peroxidase-conjugated antibodies (Dako, Carpinteria, CA). Chemiluminescence films were analyzed using a GS-700 imaging densitometer and Multi-Analyst software (Bio-Rad).

Surface expression of CD4 was measured by flow cytometry using fluorescein-conjugated Leu3a monoclonal antibody and a FACStar Plus cytometer (BD Pharmingen). 10,000 cells were acquired at a flow rate of 500–1000 particles per s.

**HIV env Expression Vectors**—The following HIV molecular clones were used to create the env expression vectors in pCDNA3.1/myc-HisA+ (Invitrogen): p89.6 (17) from R. G. Collman, University of Pennsylvania School of Medicine, Philadelphia, PA; pSVIII-92UG024.2 (18) from B. Hahn through the National Institutes of Health AIDS Reference and Reagent Program; and QH1521.34 (19) from M. L. Greenberg, Department of Surgery, Duke University Medical Center, Durham, NC.

**Cell-Cell Fusion Assay**—Luciferase reporter assays for cell-cell fusion were performed as described (20) with minor modifications. HEK Tet-On target cells expressing comparable, doxycycline-dependent levels of wild-type or C130A,C159A mutant CD4 were co-transfected with 0.25 μg of pMTLuc (a bacteriophage T7 promoter-driven firefly luciferase expression vector) (21) and 0.25 μg of pc.XCXR4 or pc.CCR5 (obtained from Dr. N. Landau (22) through the National Institutes of Health AIDS Research and Reference Reagent Program) and incubated for 48 h at 37 °C and 5% CO\(_2\). HEK 293T effector cells were co-transfected with 0.25 μg of env vectors and 0.125 μg of pCAGT7 (a T7 RNA polymerase expression vector) (23) and incubated for 48 h at 37 °C and 5% CO\(_2\). The transfected target cells were washed twice in serum-free medium, harvested with phosphate-buffered saline containing 0.05% EDTA, resuspended in 1 ml of complete medium, plated onto the same number of effector cells that had been washed twice with phosphate-buffered saline, and co-cultured for 24 h at 37 °C and 5% CO\(_2\). The fusion of effector and target cells was determined using the Luciferase Reporter Assay system (Promega, Madison, WI) and expressed as relative light units. Surface expression of X4 and R5 chemokine receptor was measured by flow cytometry using phycoerythrin-conjugated CD184 and CD195 monoclonal antibodies (BD Pharmingen), respectively, and a FACStar Plus cytometer.

**Reporter Virus Entry Assay**—Luciferase reporter viruses containing pseudotyped env were produced by co-transfecting HEK 293T cells (250,000 cells per ml in 6-well Nunc cul-
ture plates) with 1 µg of env vector and 1 µg of provirus expression vector pNL4.3.Luc.R-E in which the Nef gene has been replaced with luciferase (obtained from Dr. N. Landau (24)) through the National Institutes of Health AIDS Research and Reference Reagent Program. Virions were harvested from the conditioned medium at 72 h after transfection, filtered through 0.45-µm filters, and stored at −80 °C until use. HEK 293T cells (250,000 cells per ml in 12-well Nunc culture plates) were co-transfected with 1 µg of pcDNA3/wt CD4 or pcDNA3/C130A,C159A CD4 and 1 µg pc.CXCR4 and incubated for 24 h at 37 °C and 5% CO₂. Viral supernatant (0.25 ml in a total volume of 0.5 ml of complete medium) was added to the transfected cells and incubated for 1 h at 37 °C and 5% CO₂. Cells were washed twice in serum-free medium and then treated with 0.05% trypsin and 0.5 mM EDTA for 5 min at 37 °C to minimize trapped virus (25). Cells were washed a further two times in serum-free medium and incubated with complete medium for a further 24 h. Viral entry was determined using the Luciferase Reporter Assay system and expressed as relative light units.

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

RESULTS

Redox Potential of the CD4 Domain 2 Disulfide Bond—The redox potential of the Cys¹³⁰-Cys¹⁵⁹ disulfide was determined using oxidized and reduced DTT. Recombinant CD4 domain 2 (residues 125–202) was incubated with increasing ratios of reduced to oxidized DTT and the protein resolved on SDS-PAGE. The ratio of reduced to oxidized protein was determined by differential staining by colloidal Coomassie (Fig. 1A). A redox potential of −241 mV was calculated using the Nernst equation (Fig. 1B).

Mechanism-based Kinetic Trapping of the Cys¹³⁰-Cys¹⁵⁹ Disulfide Bond with Human Thioredoxin 1—The CD4 bond with a redox potential of −241 mV was predicted to be reduced by thioredoxin, which has a redox potential of −270 mV (26). This was confirmed using a thioredoxin-trapping mutant, in which the active site cysteine (Cys³⁵) that normally resolves the mixed disulfide has been mutated to serine (15, 16). Replacement of Cys³⁵ with serine stabilizes the covalent intermediate. Incubation of CD4 domain 2 with wild-type thioredoxin or C35S trapping mutant resulted in a complex of domain 2 with the trapping mutant but not with wild-type thioredoxin (Fig. 2). The complex was disulfide-linked as it resolved upon reduction with 20 mM DTT.

Inducible Cell Expression of Wild-type and C130A,C159A Mutant CD4—HEK Tet-On cells were stably transfected with wild-type or C130A,C159A mutant CD4, and expression was induced with doxycycline. To achieve comparable expression of the CD4s, wild-type cells were induced with 0.5 µM doxycycline and mutant cells with 2 µM doxycycline (Fig. 3, A and B). Expression of both proteins was doxycycline-dependent, and there was no expression of CD4 in cells transfected with empty vector. Surface expression of X4 or R5 chemokine receptor was comparable in cells expressing either wild-type or mutant CD4 (Fig. 3C).

Flow cytometry detection of wild-type and mutant CD4 was the same whether Leu3a antibody or fluorescein-conjugated gp120 was used (data not shown). In an earlier study, binding of Leu3a antibody to fibrosarcoma cells expressing wild-type or C130A,C159A mutant CD4 was comparable, and gp120 binding to CEMT4 cells was unaffected by alkylation of reduced CD4 with a biotin-linked maleimide (9). These results indicate that eliminating the domain 2 disulfide bond does not impair binding of ligands to domain 1.

Elimination of the CD4 Domain 2 Disulfide Bond Enhances HIV env-mediated Cell-Cell Fusion—The HEK Tet-On cells expressing comparable levels of wild-type or C130A,C159A
mutant CD4 described above were co-transfected with bacteriophage T7 promoter-driven firefly luciferase and X4 or R5 chemokine receptor. They were fused with HEK 293T cells co-transfected with T7 RNA polymerase and primary HIV env for 1 h. Clade B (89.6 (R5X4) and QH1521.34 (R5X4)) HIV envs were employed for the studies. The fusion of effector and target cells was determined by luciferase activity. Elimination of the CD4 domain 2 disulfide bond enhanced fusion with both HIV env using either X4 or R5 chemokine receptor from 1.9- to 3.7-fold \((p < 0.001; \text{Fig. 4})\).

Elimination of the CD4 Domain 2 Disulfide Bond Enhances HIV Entry—The HEK Tet-On cells expressing CD4 were not infectable by HIV reporter viruses, for unknown reasons. We instead measured reporter virus entry into HEK 293T cells transiently transfected with CD4 and chemokine receptor. Luciferase reporter viruses containing pseudotyped env were produced by co-transfecting HEK 293T cells with Clade D 92UG024 (X4) HIV env and a provirus in which the Nef gene has been replaced with luciferase. Reporter virus was added to HEK 293T cells co-transfected with wild-type or C130A,C159A mutant CD4 and X4 chemokine receptor for 1 h, unbound virus removed, and the cells incubated for a further 24 h. Viral entry was determined by luciferase activity.

FIGURE 3. Inducible cell expression of wild-type and C130A,C159A mutant CD4. CD4 expression was induced with doxycycline in HEK Tet-On cells stably transfected with wild-type (0.5 \(\mu\)M doxycycline) or C130A,C159A mutant (2 \(\mu\)M doxycycline) protein. A, Western blot of HEK Tet-On cell lysates showing comparable, doxycycline-dependent expression of wild-type and mutant CD4 protein. A blot for GAPDH is shown as loading control. The positions of molecular weight markers are shown on the left. B, Flow cytometry of HEK Tet-On cells showing comparable, doxycycline-dependent surface expression of wild-type (mean fluorescence = 226) and mutant (mean fluorescence = 207) CD4 protein. CD4 expression in cells transfected with empty vector is shown as control. C, Flow cytometry of HEK Tet-On cells showing comparable surface expression of X4 or R5 chemokine receptor in cells expressing either wild-type or mutant CD4 protein. X4 and R5 mean fluorescence is 734 and 1282, and 2942 and 2346 in cells expressing wild-type or mutant CD4 protein, respectively. Nil is cells transfected with empty chemokine vector.

FIGURE 4. Elimination of the CD4 domain 2 disulfide bond enhances HIV env-mediated cell-cell fusion. Fusion of HEK Tet-On target cells expressing comparable levels of wild-type or C130A,C159A mutant CD4 and X4 or R5 chemokine receptor with HEK 293T effector cells expressing either 89.6 or Q1521.34 HIV env is shown. Fusion is reported as luciferase units. The bars and errors are the mean \pm S.D. of four experiments. ***, \(p < 0.001\).
Expression of the C130A,C159A mutant CD4 was usually lower than expression of wild-type protein in this system. Surface expression of wild-type CD4 varied from 14% to 88% of cells in 23 separate experiments based on gated mean fluorescence values, and surface expression of the C130A,C159A mutant varied from 5% to 50% of cells in 21 separate experiments. We have selected experiments where cells expressed comparable levels of wild-type and mutant CD4 (Fig. 5A). Correction of the assay data for different expression of wild-type and mutant CD4 could possibly bias the result. Elimination of the domain 2 disulfide bond increased entry of the reporter viruses 3-fold in two separate experiments (p < 0.05 and p < 0.001, Fig. 5B).

DISCUSSION

Monoclonal antibody studies support a conformational change in CD4 following binding of HIV env to domain 1. Bachelder et al. (27) immunized an HIV-infected individual with human soluble CD4 and panned a recombinant Fab library constructed from the bone marrow with CD4-gp120 complexes. A Fab clone was isolated that recognizes cell surface CD4 only after incubation with a recombinant HIV gp120 or HIV virions. A murine monoclonal antibody, 5A8, has also been characterized that binds to domain 2 of CD4 and blocks HIV infection and syncytium formation of CD4+ cells (28) but not binding of HIV or gp120 to CD4 (29). Antibodies that interact with domains 3 and 4, in contrast, had little or no effect on HIV infection, syncytium formation, or CD4-induced conformational changes in HIV env (29). Two other monoclonal antibodies raised against the CD4-gp120 complex also block env-mediated cell-cell fusion without impairing binding of gp120 to CD4 (30, 31). The epitopes for these antibodies have not been mapped, but it is likely that they are recognizing a gp120-induced conformational change in CD4. These results support a virus-induced conformational change in cell surface CD4.

The nature of this conformational change is unknown, although residues located in domain 2 have been implicated in the change. Domain 2 residues Pro121, Pro122, Gly123 (32), and Asn164 (33) were found to be important for a high affinity CD4-gp120 interaction, and antibodies that bind to epitopes that encompass these residues block HIV syncitia formation and/or infection (29, 33–37). These residues are also within two stretches of domain 2 amino acids, 120–127 or 163–166, that from molecular modeling studies have been proposed to constitute a hinge that bends upon HIV binding to domain 1 (12). We suggest that reduction of the Cys130-Cys159 domain 2 disulfide bond may mediate the conformational change in CD4 that is associated with HIV infection (27–29).

To define further the properties of the CD4 domain 2 disulfide, we have determined its redox potential. A standard redox potential of −241 mV was calculated for the Cys130-Cys159 bond using recombinant domain 2 protein. This is in the range of the standard potentials of three other –RHStaple disulfides; −229 mV for the Cys103-Cys109 DsbD bond (38), −278 mV for the Cys186-Cys209 tissue factor bond,3 and −261 mV for the Cys149-Cys202 bond engineered into green fluorescent protein (39). Thioredoxin with a redox potential of −270 mV (26) is predicted, therefore, to be able to cleave the Cys130-Cys159 CD4 bond. Protein disulfide isomerase with a redox potential of −175 mV (40), on the other hand, does not cleave the CD4 bond (data not shown). Cleavage of the CD4 Cys130-Cys159 bond by thioredoxin was confirmed using a mechanism-based trapping mutant (15, 16).

The functional significance of the Cys130-Cys159 bond for HIV entry and env-mediated cell-cell fusion was tested using HEK Tet-On cells expressing comparable levels of wild-type or C130A,C159A mutant CD4. Ablation of the domain 2 disulfide bond increased HIV entry and env-mediated cell-cell fusion.

3 H. P. H. Liang and P. J. Hogg, unpublished observations.
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Ablation of the domain 2 disulfide bond destroys co-receptor activity (13), indicating that the disulfide-linked domain 2 disulfide bond. Strategies to promote dimerization of CD4 should impair HIV infection and favor co-receptor activity.

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