Disulfide Reduction in CD4 Domain 1 or 2 Is Essential for Interaction with HIV Glycoprotein 120 (gp120), which Impairs Thioredoxin-driven CD4 Dimerization*

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Received for publication, December 10, 2013, and in revised form, February 12, 2014. Published, JBC Papers in Press, February 18, 2014, DOI 10.1074/jbc.M113.539353

Background: Interaction between HIV gp120 and cell CD4 initiates viral infection of host cells.

Results: Only CD4 with reduced disulfides in domain 1 or 2 binds gp120, which inhibits thioredoxin-dependent CD4 dimerization.

Conclusion: Cell surface oxidoreductases may prime CD4 for gp120 engagement, and impairment of redox-driven CD4 dimerization by gp120 may compromise CD4 function.

Significance: Redox-dependent isomerization of CD4 is critical for HIV entry.

Human CD4 is a membrane-bound glycoprotein expressed on the surface of certain leukocytes, where it plays a key role in the activation of immunostimulatory T cells and acts as the primary receptor for human immunodeficiency virus (HIV) glycoprotein (gp120). Although growing evidence suggests that redox exchange reactions involving CD4 disulfides, potentially catalyzed by cell surface-secreted oxidoreductases such as thioredoxin (Trx) and protein disulfide isomerase, play an essential role in regulating the activity of CD4, their mechanism(s) and biological utility remain incompletely understood. To gain more insights in this regard, we generated a panel of recombinant 2-domain CD4 proteins (2dCD4), including wild-type and Cys/Ala variants, and used these to show that while protein disulfide isomerase has little capacity for 2dCD4 reduction, Trx reduces 2dCD4 highly efficiently, catalyzing the formation of conformationally distinct monomeric 2dCD4 isomers, and a stable, disulfide-linked 2dCD4 dimer. Moreover, we show that HIV gp120 is incapable of binding a fully oxidized, monomeric 2dCD4 in which both domain 1 and 2 disulfides are intact, but binds robustly to reduced counterparts that are the ostensible products of Trx-mediated isomerization. Finally, we demonstrate that Trx-driven dimerization of CD4, a process believed to be critical for the establishment of functional MHCII-TCR-CD4 antigen presentation complexes, is impaired when CD4 is bound to gp120. These observations reinforce the importance of cell surface redox activity for HIV entry and posit the intriguing possibility that one of the many pathogenic effects of HIV may be related to gp120-mediated inhibition of oxidoreductive CD4 isomerization.

* This work was supported by the South African Medical Research Council, the National Research Foundation, the Polioymetylitis Research Foundation, the Carnegie Foundation, and the South African HIV/AIDS Research Platform of the Department of Science and Technology.

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2 The abbreviations used are: D1, domain 1; TCR, T cell receptor; PDI, protein disulfide isomerase; Trx, thioredoxin; TR, thioredoxin reductase; IAM, iodoacetamide; MPB, N-(3-maleimidopropionyl)biocytin; gp120, glycoprotein 120.
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dide bears the classical features of the so-called “allosteric” or “functional” disulfide bond (12). This type of bond is rare in proteins, and the pronounced torsional strain on the disulfide is thought to enable its relatively facile reduction, triggering local or global conformational changes that may be functionally significant.

The biological importance of CD4 allostericity inferred from this is poorly understood, although several lines of evidence suggest that redox-dependent isomerization of CD4 plays a role in the context of both HIV-1 gp120 receptor engagement and in the formation of stably associated CD4-TCR-MHCII complexes. The interaction of CD4 with HIV-1 gp120 appears to involve CD4 monomers with CD4 dimerization-defective mutants (13), and specifically reduced monomeric forms of CD4 (14), supporting higher levels of HIV infection in cell culture. In contrast, it has been shown that CD4 oligomerization is necessary for stable MHCII binding (9) and that effective T cell activation requires CD4 dimer formation (15, 16), a process that evidence suggests involves redox exchanges in CD4 cysteines (11, 17, 18).

How might redox-dependent isomerization of CD4 be achieved, and what role does this play in the mechanism of CD4-dependent HIV infection? There is now an abundance of data demonstrating the effects of manipulating the activities of cell surface-active oxidoreductases such as protein disulfide isomerase (PDI) and thioredoxin (Trx) on HIV infection in vitro (19–23), although considerable debate remains on the biological utility and physiological relevance of such effects and the extent of involvement of these factors in vivo. Furthermore, and complicating the elucidation of the biochemical importance of these enzymes for the mechanism of HIV entry, are the observations that gp120, itself containing nine disulfide bonds, is also a substrate for PDI, Trx-, and glutaredoxin-1-mediated reduction in vitro (19, 24, 25) and that inhibitory antibodies to PDI or Trx have different effects in the context of macrophage- or lymphocytic HIV infection (26).

In this study, we show that although PDI has very low capacity for CD4 reduction, Trx reduces 2dCD4 disulfides robustly when activated by thioredoxin reductase (TR) in vitro, lending further support to the widely-held notion of Trx as the more likely physiological reductant of CD4. We further demonstrate that HIV gp120 binds specific reduced isomers of 2dCD4, the representation of which are significantly increased through reduction by Trx. In contrast, gp120 is completely incapable of binding a fully oxidized, monomeric CD4 in which both D1 and D2 disulfide bonds are intact. Moreover, Trx-mediated dimerization of CD4, which potentially plays a pivotal role in the establishment of functional TCR-CD4-MHCII complexes, is impaired when CD4 is bound to gp120. These data establish an additional possible rationale for the importance of cell-surface oxidoreductase activity in HIV-1 infection and simultaneously put forward another potentially important pathogenic consequence of CD4-gp120 binding for consideration.

**EXPERIMENTAL PROCEDURES**

*Vectors and Proteins*—DNA cassettes encoding the first two amino-terminal domains of wild-type human CD4 (2dCD4-WT, residues 1–183) and the following four Cys/Ala variants, C159A, C16A/C84A, C130A/C159A, and C16A/C84A/C130A/C159A, in-frame with C-terminal His6 fusion tags were synthesized by GENEART (Regensburg, Germany) following codon optimization for expression in Escherichia coli. The recombinant 2dCD4 variants were expressed and purified by standard denaturing metal chelate affinity chromatography and refolded using oxidative refolding protocols according to methods described previously (27). Recombinant HIV-1 gp120 (BaL) was expressed in a stably transfected HEK293 cell line previously generated in our laboratory and purified by two-step affinity and size-exclusion chromatography. Following purification, both proteins were exhaustively dialyzed into phosphate-buffered saline (PBS, pH 7.4), concentrated, filter-sterilized, and stored in aliquots at −80°C. The proteins were analyzed by reducing or non-reducing SDS-PAGE using precast 4–12% gradient NuPAGE gels (Invitrogen). Prior to gel loading, the samples were treated with LDS loading buffer with or without DTT (50 mM) and incubated at room temperature for 10 min. Iodoacetamide (IAM, Sigma-Aldrich) was then added to a final concentration of 50 mM. Gels were stained with Coomassie Blue R-250.

**2dCD4 MPB Labeling Assay**—To detect the free thiol content of 2dCD4-WT isomers, purified recombinant 2dCD4-WT was labeled with N-(3-maleimidopropionyl)biocytin (MPB, Sigma-Aldrich). Reactions contained 15 μM recombinant 2dCD4-WT and 150 μM MPB in PBS (pH 7.4), and these were incubated at room temperature for 1 h. Unconjugated MPB was removed by centrifugation through desalting columns containing Sephadex G10 equilibrated in PBS. Samples (100 ng) were then resolved by non-reducing SDS-PAGE and transferred to nitrocellulose membranes by standard Western blotting procedures. The membranes were probed with an HRP-conjugated anti-streptavidin antibody or an anti-His6 antibody (Qiagen, Germany) followed by a secondary HRP-conjugated antimouse IgG (GE Healthcare). Bound antibodies were visualized by standard chemiluminescence procedures using a ChemiDoc imaging instrument and Quantity One software (Bio-Rad).

*PDI/Trx Reduction Assay*—Reduction of disulfide-bonded insulin is commonly used to measure the efficiency of disulfide reductase activity (28). We used a similar approach to assess whether PDI and/or Trx catalyzed the reduction of 2dCD4-WT. For the Trx reaction, 50 μM 2dCD4-WT, insulin (Novo Nordisk) or BSA (Roche Applied Science) was incubated with 0.2 mM NADPH (Calbiochem), 2 mM EDTA, and 4 μM human Trx1 (IMCO Corp., LTD AB, Sweden) in 0.1 M potassium phosphate (pH 7.4). The 100-μl reaction was started with 7 nM HIV gp120 and/or insulin, or BSA (Roche Applied Science) was incubated with 0.2 mM NADPH (Calbiochem), 2 mM EDTA, and 4 μM human Trx1 (IMCO Corp., LTD AB, Sweden) in 0.1 M potassium phosphate (pH 7.4). The 100-μl reaction was started with 7 nM mammalian thioredoxin reductase (TR). For the PDI reduction reaction, 2dCD4-WT, insulin, or BSA were incubated with 4 μM PDI (Takara Bio., Inc., Japan), 2 mM EDTA in 0.1 M potassium phosphate, and the 100-μl reaction was started with 100 μM DTT (Sigma-Aldrich). All reactions were prepared in triplicate, and control reactions of either 2dCD4-WT alone or 2dCD4-WT with no activator (TR or DTT for the Trx and PDI reactions, respectively) were performed in parallel. A550 readings were taken every 10 s for 10 min.

*Trx-mediated 2dCD4 Isomerization Analysis*—To analyze the products of oxidoreductase-mediated 2dCD4 disulfide reduction, 2dCD4-WT or purified 2dCD4-gp120BaL complexes...
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We previously reported the production of a recombinant protein containing the first two N-terminal domains of human CD4 (2dCD4-WT) using an E. coli-based expression system (27). Following extraction from inclusion bodies, purification by nickel-nitrilotriacetic acid affinity chromatography and refolding under conditions that promote disulfide bond formation, functional 2dCD4-WT can be prepared to apparent homogeneity, resolving as a single band of ~28 kDa when analyzed by denaturing, reducing SDS-PAGE (Fig. 1A). When analyzed under non-reducing conditions, which would maintain disulfide bond integrity, the purified 2dCD4-WT resolves into two distinct isomers that have increased mobility compared with the fully reduced isofrom (Fig. 1A). In general, reduction of disulfide bonds has been shown to decrease relative electrophoretic mobility of proteins by increasing the hydrodynamic volumes of the resultant structures (29), and this effect has previously been demonstrated on full-length CD4 expressed in mammalian cells (30). Accordingly, we reasoned that the differentially resolved 2dCD4-WT isomers represented distinct redox intermediates containing either both domain cysteines in the oxidized disulfide state or reduced cysteine pairs in either D1 or D2 or both.

To assign oxidation states of the cysteine pairs within these isomers, we generated a panel of identical 2dCD4 proteins containing double Cys/Ala substitutions in each domain (2dCD4-C16A/C84A and 2dCD4-C130A/C159A), as well as a variant in which all four cysteines were mutated to alanine (2dCD4-ΔA). As expected, under reducing, denaturing conditions each variant migrated as a single band of ~28 kDa (Fig. 1B, top panel). When the samples were alkylated with IAM and analyzed under non-reducing conditions, each variant resolved into one to three distinct isomers ranging from an estimated 22–28 kDa (Fig. 1B, bottom panel). Consistent with its inability to form disulfide bonds in D1 and D2, 2dCD4-ΔA migrates at a position identical to that of reduced 2dCD4-WT establishing the species at 28 kDa as a fully reduced 2dCD4 isofrom in which both domain cysteines are reduced. In contrast, the predominant species found in preparations of 2dCD4-C130A/C159A, which is capable of forming only the D1 disulfide bond, is found at ~25 kDa, suggesting that the isofrom which migrates to this position in 2dCD4-WT contains a stable disulfide in D1 and reduced cysteines in D2. Interestingly, the mobility of the main isofrom found in the domain, one double Cys/Ala variant, 2dCD4-C16A/C84A, at 28 kDa identical to 2dCD4-ΔA and fully reduced 2dCD4-WT, implies that 2dCD4-C16A/C84A is unable to form the D2 disulfide, perhaps through the absence of cooperative contributions made by the D1 cysteines during folding of this protein. However, a previous study demonstrated marked differences in the effects that abolition of the D1 and D2 disulfides have on the mobility of full-length CD4 expressed in HeLa cells (30). In this study, it was shown that the migration rate of a full-length CD4 containing alanine mutations at Cys-16 and Cys-84 was pronouncedly retarded, whereas corresponding alanine substitutions at Cys-130 and Cys-159 had no effect in this regard. Clearly the context of these mutations, whether present in a full-length or two-domain CD4, is an important determinant of the effect they have on the structures, hydrodynamic volumes, and ultimately the migration rates of these molecules. Thus, we propose, and, as the gp120-binding data shown in Fig. 4 appear to confirm, that 2dCD4 in which both domain disulfides are reduced, and 2dCD4, in which only the domain 1 disulfide is reduced, each migrate with the same apparent molecular mass of 28 kDa.

Most importantly, only the 2dCD4-WT preparation contained the fast-migrating isofrom at ~22 kDa (Fig. 1B, bottom panel). In keeping with the exclusive ability of this construct to...
form both domain disulfides, we propose that this band represents a 2dCD4 in which both domain cysteines exist in the disulfide state. Because a theoretical argument could be made that this isoform of 2dCD4-WT might contain interdomain disulfide bonds, perhaps formed through kinetic end points encountered during the refolding process, we generated a 2dCD4 mutant containing a single Cys/Ala substitutions in domain 2 (2dCD4-C159A). Trans-domain oxidation between Cys-130 and either D1 cysteine in this molecule would be expected to have a dramatic effect on either the solubility or conformation, and thus mobility, of the resultant polypeptide. Gel analysis indicated that this is indeed unlikely to be the case because 2dCD4-C159A exists predominantly as a soluble isoform containing an intact D1 disulfide, as suggested by the identity of the mobility between this and 2dCD4-C130A/C159A (Fig. 1C).

To gain further confirmation regarding the proposed assignments of the oxidation states of the cysteines within each redox isomer, we used MPB to label any free thiols present on the various isomers present in the 2dCD4-WT preparation. Following Western transfer of the MPB-labeled protein, probing with anti-His detected both species at 22 and 25 kDa (Fig. 1D, left panel), whereas only the 25-kDa isoform and small amount of a fully reduced, 28-kDa isoform, which is occasionally present in 2dCD4-WT preparations (for examples, see Figs. 3–5) and not detectable with the anti-His probe, was detected with streptavidin-HRP (Fig. 1D, right panel). Taken together, these results provide compelling evidence that recombinant 2dCD4-WT exists predominantly as two distinct isomeric species, one of which contains intact disulfides in both D1 and D2. For ease of reference, we henceforth refer to the 2dCD4-WT species migrating at 28, 25, and 22 kDa under non-reducing conditions as 2dCD4R1, 2dCD4R2, and 2dCD4Ox, respectively.

**PDI- and Trx-mediated 2dCD4 Reduction**—We next set out to assess whether 2dCD4 disulfides were targets for enzymatic reduction by Trx and/or PDI in vitro. In this assay, intermolecular disulfide exchanges between thiols formed from reductase activity results in progressive precipitation of the target protein, the extent of which, determinable by measuring solution turbidity at $A_{650}$, provides an indication of the efficiency of target protein reduction. We treated the same concentrations of recombinant insulin, commonly used as a target for assessing reductase activity (28), as a positive control for the PDI and Trx activity, and BSA, which contains 35 cysteines, to control for nonspecific effects of reduction. Under these conditions, both Trx and PDI catalyzed reduction of insulin over a 10-min reaction course (Fig. 2A, top panels), whereas only Trx reduced 2dCD4 robustly (Fig. 2A, middle panels) over the same time interval. No reduction of BSA by either Trx or PDI was detected (Fig. 2A, bottom panels), and Trx-mediated reduction was spe-
Specifically attributable to the catalytic activity of the oxidoreductase because no increase in turbidity was observed in identical reactions that omitted Trx (Fig. 2A, middle right panel). Interestingly, an increase in turbidity in PDI-treated 2dCD4 samples was noted after an extended period of incubation (>15 min, Fig. 2B), which, together with the observation that both PDI and Trx are secreted on the surfaces of human lymphocytes (31, 32), suggests that certain conditions and contexts may exist under which PDI could have catalytic effects on CD4. However, calculations of the respective redox potentials of the CD4 D2 disulfide (14) and the active site dithiols of these enzymes (33, 34) have suggested that Trx is the more likely physiological reductant of CD4, and our data, which shows substantially more efficient reduction of CD4 by Trx, via a mechanism of activation that is more realistically feasible in the cellular context, appear to be consistent with this.

Trx-mediated 2dCD4 Isomerization—We then investigated whether the robust enzymatic reduction of CD4 effected by Trx resulted in observable isomerization of the constituent 2dCD4 samples. A time-resolved enzyme assay was performed in which Trx/TR/NADPH were incubated with 2dCD4-WT for 0–60 min, and the reactions were stopped at various intervals with 50 mM IAM. Samples were then resolved by reducing (Fig. 3A, bottom panel), and non-reducing SDS-PAGE (Fig. 3A, top panel), and the relative quantities of the 2dCD4-WT isomers determined by densitometry (Fig. 3B). Incubation of 2dCD4-WT with Trx treatment resulted in a time-dependent decrease in the amount of 2dCD4Ox, which was completely ablated after 10–30 min of enzymatic reduction. At the same time, 2dCD4R2 and a species of ~50 kDa, most likely representing a disulfide-bonded 2dCD4-WT dimer (2dCD4-WT<sup>D</sup>) are formed. Although the quantity of CD4 dimer does not change significantly beyond this point, the amount of 2dCD4R<sup>R2</sup> increases progressively for the duration of the reaction. Conversely, although 2dCD4R<sup>R1</sup> remained at near constant levels for the first 10 min of treatment, the levels of this isomer declined toward the end of the reaction cycle. Considering the commensuration in the decline of 2dCD4R<sup>R1</sup> and increase in 2dCD4R<sup>R2</sup> from 10 to 60 min, and the observation that these changes continue long after 2dCD4Ox has been depleted and steady-state levels of 2dCD4<sup>D</sup> achieved, these findings imply that exchanges involving the D1 disulfide of CD4 may also be an important component of Trx-mediated CD4 isomerization.

To confirm that these isomerization events were exerted specifically through the catalytic function of Trx, control reactions were done at 0 and 60 min and included 2dCD4-WT alone, 2dCD4-WT in the presence of reducing equivalent only (TR-NADPH) and 2dCD4-WT in the presence of Trx alone. In each of these cases, no change in the composition of native 2dCD4-WT isomers was observed after 60 min. As final confirmation that the isomerization was related fundamentally to changes in the oxidation states and bonding patterns of 2dCD4-WT cysteines, and that the dimeric 2dCD4 species was in fact disulfide-bonded, the Trx-treated samples were resolved under reducing conditions. Under these conditions, the isomerized 2dCD4s resolved as a single, fully-reduced protein of 28 kDa (Fig. 3A, bottom panel). An acknowledged limitation of this assay is its inability to define the exact sequence of...
isomerization events, for example, which species represent the immediate precursors for 2dCD4 dimer formation, and ongoing experiments using various 2dCD4 Cys/Ala mutants should provide further insights in this regard. However, taken together, the data reported here allow us to conclude that 2dCD4Ox is a substrate for Trx-mediated reduction, which may involve disulfides located in both D1 and D2, and this activity results in the formation of a disulfide-bonded 2dCD4 dimer.

2dCD4 Isomer-specific gp120 Binding—Previous studies have shown that reduced forms of CD4 are the preferred receptor for HIV-1 gp120 (14). To gain further insights in this regard, we applied a chromatographic approach to analyze to what extent the constituent isomers within 2dCD4-WT, 2dCD4-C16A/C84A, and 2dCD4-C130A/C159A are capable of binding HIV-1 gp120. Remarkably, both 2dCD4-C16A/C84A and 2dCD4-C130A/C159A, but neither 2dCD4Ox nor 2dCD4-CΔA, bound gp120 robustly (Fig. 4B).

To check whether the 2dCD4 variants interacted with gp120 through specific, functional interaction, we performed an ELISA assay to establish whether their binding resulted in the exposure of coreceptor (CCR5/CXCR4) binding sites on gp120. These sites (so-called CD4-induced (CD4i) epitopes) are efficiently exposed on gp120 only when the latter is engaged with CD4 and can be detected by using CD4i-specific antibodies (of which the monoclonal antibody 17b is one of the best characterized). Recombinant gp120 was allowed to react with the 2dCD4 variants at a range of concentrations, and the complexes were captured on a gp120-antibody-coated microtitre plate. The captured complexes were then probed with 17b antibody. As expected, 2dCD4-WT, -C16A/C84A, and C130A/C159A, but not 2dCD4-CΔA, resulted in a robust, dose-dependent induction of the 17b-binding site on gp120 (Fig. 4C), confirm-
ing the functionality of its interactions with the 2dCD4 isomers. Taken together, these data are consistent with previous reports demonstrating the preference of gp120 for reduced forms of CD4 and expand on these with two intriguing observations: that CD4 requires reduction of either the domain 1 or domain 2 disulfide (but not both) to bind gp120 and is impaired in doing so when both domain disulfides are intact.

To gain further insights into the impact of disulfide ablation in D1 or D2 on 2dCD4 structure, we then performed far-UV CD spectroscopy on 2dCD4-WT, -C16A/C84A, and -C130A/C159A, and compared these with the CD spectrum predicted for the 2dCD4 crystal structure (Protein Data Bank code 1CDH) using the DichroCalc CD spectra prediction algorithm (Fig. 5) (35). Relative to the curve predicted for 1CDH, which is typical of classical \( \beta \)-sheeted secondary structure with an ellipticity minimum of approximately \( 40 \times 10^{-3} \text{ deg.cm}^2/\text{dmol} \) at 220 nm, the curve for 2dCD4-WT, which is a composite of fully and partially oxidized isoforms, is leftward-shifted (min \( \theta_{MRW} \times 10^{-3} \) of \( \sim -60 \times 10^{-3} \text{ deg.cm}^2/\text{dmol} \) at 212 nm). The magnitude of the ellipticity minimum, and the wavelength at which this occurs for 2dCD4-C16A/C84A and 2dCD4-C130A/C159A, are further decreased to \( -75 \times 10^{-3} \) and \( -79 \times 10^{-3} \text{ deg.cm}^2/\text{dmol} \) at 206 and 204 nm, respectively. This progressive decrease in wavelength and magnitude of the ellipticity minimum correlates inversely with total disulfide content and is consistent with a relaxation in the ordered \( \beta \)-sheeted structure (Fig. 5A) that disulfide reduction in each 2dCD4 domain is likely to effect.

**gp120 Inhibits Oxidoreductase-mediated Dimerization of 2dCD4**—Oligomerization of CD4 is now known to be a critical component of establishing functional CD4/TCR-MHCII complexes (36), which are required for efficient transduction of activation signals transmitted to T cells by MHCII-associated antigenic peptides. Failure to achieve such antigen presentation complexes results in T cell anergy and apoptosis (2). By virtue of the critical role that CD4 \( ^+ \) T cell activation plays in mediating effective humoral immune responses, corruption of normal CD4 function, potentially mediated by virus-associated gp120 or soluble gp120 known to be shed from the viral envelope into the blood of HIV-infected individuals, may make a significant contribution to the pathogenic consequences of HIV infection. Considering this, we investigated whether one of the corruptive effects of gp120 binding could involve inhibition of oxidoreductase-driven dimerization of CD4. To check this, we treated equal amounts of gp120-bound and unbound 2dCD4 with Trx and analyzed the reaction products by non-reducing SDS-PAGE (Fig. 6A) and densitometry (Fig. 6B). Although unliganded 2dCD4 undergoes measurable Trx-mediated reduction

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**FIGURE 4. 2dCD4 isomer-specific gp120 interaction.** A and B, recombinant 2dCD4-WT was incubated with purified recombinant HIV-1 gp120 \( ^{\text{BaL}} \) (left panel) for 1 h at room temperature, and the gp120-bound and unbound isomers were fractionated by FPLC. The samples were treated with IAM (50 mM), concentrated, and resolved by non-reducing SDS-PAGE. B, 2dCD4-WT and the indicated Cys/Ala variants were incubated with gp120 \( ^{\text{BaL}} \) and the gp120-bound proteins purified and analyzed by non-reducing SDS-PAGE. C, gp120 –2dCD4 variant complexes were captured on an anti-gp120 antibody-coated microtiter plate and then probed with the monoclonal antibody 17b to detect induction of the CD4-induced epitope (CD4i) on gp120. The assays were performed in triplicate in three independent experiments, and representative data are shown. Error bars represent S.D. from average \( A_{450} \) levels in one experiment.
and dimerization, we found that gp120-bound 2dCD4 has significantly compromised capacity for isomerization effected by Trx. In summary, the data reported in this experiment allude to inhibitory effects mediated by gp120 on oxidoreductase-mediated isomerization of CD4, and this may represent one of the mechanisms by which gp120 corrupts important CD4-dependent signal transduction events.

DISCUSSION

An abundance of data has now demonstrated the deleterious effect of inhibiting cell surface oxidoreductase activity on HIV infection, implicating redox exchange reactions in the mechanism of HIV entry. For example, inhibition of cell surface redox activity with the membrane-impermeant sulfhydryl reagent 5,5'-dithiobis(2-nitrobenzoic acid) impairs HIV-1 infection in cell culture, as do specific inhibitors of PDI such as bacitracin, anti-PDI antibodies (20), and PDI siRNA (22), and similar effects have now been shown for Trx (11) and other important cellular reductases such as glutaredoxin-1 (24). Considerable debate remains on the physiological relevance of these observations because, in addition to facilitating HIV replication in cell culture, all three enzymes have been shown to be expressed on the surface of mammalian cells (31, 32) and to reduce disulfide bonds of gp120 in vitro (11, 24, 25). Contributing to the controversy are questions on how these enzymes would source the reducing equivalents required to charge their active sites in the oxidizing environment of the extracellular milieu. Indeed, in the PDI assays applied in this study, enzyme activation was achieved with 100 μM DTT, a more potent reductant at substantially higher molar concentration than what reasonable estimates suggest would typically be present on the cell surface (roughly 10 μM GSH equivalent) (24). The Trx relay system we employed, used to charge Trx with TR and NADPH, reconstitutes the components of a physiologically relevant redox system and mediates significantly more efficient reduction of CD4, providing further support to previous proposals that Trx is a good candidate for CD4 reduction in the cellular context. However, for the time being at least, the exact oxidoreductases involved and the mechanism(s) by which such systems secure adequate supplies of reduced co-factors (e.g. NADPH in the case of Trx/TR) to carry out their catalytic functions efficiently at the cell surface remain incompletely understood. Studies on the existence of membrane-embedded reduction systems should provide further insights in this regard.

Given an appropriate means of activation, however, our data show unequivocally that Trx is capable of robust reduction of CD4. In general, its exertion results in the rapid depletion of an oxidized monomeric isomer of the protein (2dCD4Ox) and the progressive, commensurate formation of reduced counterparts, a process that leads either directly or via several intermediate steps to the production of a disulfide-linked 2dCD4

FIGURE 5. Secondary structure analysis of 2dCD4-WT, -C16A/C84A and -C130A/C159A. A, ribbon representation of two-domain CD4 (2dCD4, Protein Data Bank code 1CDH), showing the extensive β-sheeted secondary structures within domains 1 and 2, with respective inter- and intra-sheet disulfide bonds (Cys10-Cys84 and Cys130-Cys159) shown in space-filling mode (yellow). Also shown is Phe43 of D1 (green), a major constituent of the gp120-binding site on CD4. B, far-UV CD spectra of 2dCD4-WT, -C16A/C84A, and -C130A/C159A (10 μM PBS, pH 7.4) were collected using a Jasco J-810 spectropolarimeter with a 1-mm path length cuvette. Five scans were made at a scan rate of 100 nm/min, a bandwidth of 1 nm, and a response time of 2 s. All spectra were buffer-corrected, and mean residue ellipticities (deg cm² dmol⁻¹) were calculated and plotted against wavelength (nm). The pronounced leftward shift in ellipticity minima of both 2dCD4-C16A/C84A and 2dCD4-C130A/C159A suggests disruption of ordered β-sheeted structure in these proteins.
dimer. That both D1 and D2 disulfides evidently participate in these transactions is somewhat surprising, considering the documented structural characteristics of each disulfide and the preeminence given to the D2 disulfide bond as the likely mediator of CD4 allostericity in several recent reports (11, 14). However, other studies have shown that under certain conditions, CD4, in which the “non-allosteric” disulfides of D1 or D4 are reduced exists in relative abundance on the surface of cells (30), and support the notion that reduction of multiple CD4 disulfides may be functionally significant.

In this regard, there is now persuasive evidence to suggest that redox exchange reactions in the disulfides of CD4 mediate, or are at least an important component of, the structural rearrangements required for effective assembly of CD4 into TCR-MHCII antigen presentation complexes. Domain 2 Cys/Ala CD4 variants do not dimerize efficiently (11), and T cells expressing dimerization-defective CD4 cannot be activated through canonical MHCII-based presentation mechanisms (16). These data propose a rationale for the biological utility of oxidoreductive isomerization of CD4, and the ability to separate and stabilize each isomer by approaches that are conventionally applied to such endeavors obviously present fundamental technical challenges. A salient feature of both the 2dCD4 D1 and D2 disulfide mutants revealed through spectroscopic analysis is the characteristic circular dichroic spectra they generate, which are consistent with the presence of extensive irregular β-sheeted secondary structure (Fig. 5B). In contrast, 2dCD4-WT preparations (30–40% of the content of which is oxidized in D1 and D2) show far more regular and ordered β-pleating patterns (27, 41–43). This suggests that a degree of structural flexibility in CD4, potentially conferred through CD4 disulfide reduction, may be required for the initial engagement with gp120. That reduction of either domain disulfide is sufficient to enable this is an intriguing observation, the

FIGURE 6. gp120-mediated inhibition of Trx-driven CD4 dimerization. A, gp120-bound and unbound 2dCD4-WT was treated with Trx/TR/NADPH at 37 °C, and reactions were stopped at 0 and 60 min by the addition of IAM (50 mM). The samples were then resolved by non-reducing SDS-PAGE. B, the relative quantities of the indicated 2dCD4 isomers were measured by densitometry. The peak intensities of the 2dCD4 isomers are indicated in parentheses above the corresponding bars. Representative data are shown, with error bars representing the S.D. of peak intensities determined for the corresponding bands on three independent gels derived from three independent isomerization experiments.
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basis for which is currently unclear. However, the finding that 2dCD4-CΔA (in which both domain disulfides are ablated) cannot bind gp120 at all, suggests that cooperative contributions to stabilizing the overall architecture of the protein may be made by each domain and that independent reductions of their resident disulfides have related global consequences. Ongoing work in our laboratory will hopefully provide further insights in this regard. Finally, by binding reduced isomers that are ostensibly the transient precursors of CD4 dimer formation, we intuited that gp120 may corrupt redox-driven CD4 dimer assembly, which evidence now compellingly suggests is essential for effective T cell activation (15–17). We confirmed this effect using a reconstituted oxidoreduction assay, demonstrating that gp120-bound 2dCD4 experiences inefficient Trx-mediated dimerization in vitro.

In summary, we have shown that gp120 binds exclusively to recombinant forms of two-domain CD4 in which either the domain 1 (Cys\(^{16}\)–Cys\(^{84}\)) or domain 2 (Cys\(^{130}\)–Cys\(^{159}\)) disulfides are reduced. Conversely, gp120 is completely unable to bind the same protein in which both disulfides are intact. When appropriately activated, Trx reduces 2dCD4 efficiently, which leads to a rapid increase in the concentration of reduced 2dCD4 intermediates and the formation of disulfide-linked 2dCD4 dimers. Thus is proposed another potential link between the physiological requirement for redox exchange in CD4 and oxidoreductase-dependent HIV entry of host CD4\(^+\) cells. Further studies will hopefully reveal details on the structural characteristics of the CD4 redox isomers that explain their specificities for gp120, the kinetics and thermodynamics of these interactions, and establish to what extent the corruptive effects of gp120 on oxidoreductive dimerization of CD4 observed in vitro are relevant physiologically.

Acknowledgment—We thank Prof. Elias Arner (Karolinska Institutet) for the generous donation of recombinant thioredoxin reductase.

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