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Inhibition of human immunodeficiency virus infection by agents that interfere with thiol–disulfide interchange upon virus–receptor interaction

HUGUES J.-P. RYSER*†, ELINOR M. LEVY‡, RICHARD MANDEL*, AND GINO J. DISCIULLO‡

Departments of *Pathology and ‡Microbiology, Boston University School of Medicine, Boston, MA 02118

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ABSTRACT **The cell surface of mammalian cells is capable of reductively cleaving disulfide bonds of exogenous membrane-bound macromolecules (for instance, the interchain disulfide of diphtheria toxin), and inhibiting this process with membrane-impermeant sulfhydryl reagents prevents diphtheria toxin cytotoxicity. More recently it was found that the same membrane function can be inhibited by bacitracin, an inhibitor of protein disulfide-isomerase (PDI), and by monoclonal antibodies against PDI, suggesting that PDI catalyzes a thiol–disulfide interchange between its thiols and the disulfides of membrane-bound macromolecules. We provide evidence that the same reductive process plays a role in the penetration of membrane-bound human immunodeficiency virus (HIV) and show that HIV infection of human lymphoid cells is markedly inhibited by the membrane-impermeant sulfhydryl blocker 5,5′-dithiobis(2-nitrobenzoic acid), by bacitracin, and by anti-PDI antibodies. The results imply that HIV and its target cell engage in a thiol–disulfide interchange mediated by PDI and that the reduction of critical disulfides in viral envelope glycoproteins may be the initial event that triggers conformational changes required for HIV entry and cell infection. These findings suggest additional approaches to impede cell infection by HIV.**

The surface of CHO cells has a reductive function capable of cleaving disulfide bonds of various membrane-bound macromolecules, and that cleavage is inhibited by the membrane-impermeant sulfhydryl reagents 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and pCMBS (*p*-chloromercuriphenylsulfonic acid) (1, 2). In one instance, cleavage was associated with the reductive activation of diphtheria toxin (DT), a disulfide-containing protein that binds to a specific surface receptor and must undergo a reductive chain separation to exert its cytotoxicity. Both DTNB and pCMBS prevented DT cytotoxicity (2). More recent work showed (3) that monoclonal antibodies (mAbs) to protein disulfide-isomerase (PDI) and bacitracin, an inhibitor of PDI, cause similar inhibitions. PDI is an oxidoreductase capable of mediating thiol–disulfide interchange reactions. It is present mostly in the endoplastic reticulum (4) but has also been detected at the surface of mammalian cells (5, 6). Experiments based on the knowledge that DTNB inhibits DT cytotoxicity (2) and on the observation that the structure of the Sindbis virus envelope is maintained by disulfide bonds (7) showed that DTNB causes a significant inhibition of Sindbis infection of BHK cells (8, 9). The viral glycoprotein gp120 by which human immunodeficiency virus (HIV) attaches to its receptor CD4 also contains disulfide bonds (10). The events that follow HIV attachment and lead to viral infection are not yet fully understood (11). We postulated that the plasma membrane of human lymphoid cells would have a reductive function similar to that of CHO cells (3), and we reasoned that the reductive mechanism might cleave disulfides in membrane-bound gp120 and facilitate viral entry into cells. To determine whether such a process occurs and plays a role in HIV infection, the three classes of inhibitors that had been found to prevent DT cytotoxicity were tested for their possible effects on virus infection. All three inhibited the infection of cultured human lymphoid cells by HIV.

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MATERIALS AND METHODS

Cells and Materials. HIV_{IIIB} and the H9 and C8166 cell lines were obtained from Martin Hirsch (Massachusetts General Hospital, Boston) and the U937 cell line was from Tun-Hou Lee (Harvard School of Public Health, Boston). The hybridomas producing mAbs against human PDI (HP13) and rat PDI (RL90) were a gift from Charlotte S. Kaetzel (University of Kentucky College of Medicine, Lexington) (12). The mAbs were purified from ascites fluid or conditioned medium and tested for anti-PDI activity as described (3). DTNB, bacitracin, monobromotrimethylammoniobimane (Thiolyte MQ, Calbiochem), and mouse IgG were from sources as described (3). The fluorescein-labeled anti-CD4 antibody, CD4 [Leu-3a], and fluorescein isothiocyanate-conjugated irrelevant mouse IgG1 were from Becton Dickinson.

Exposure of Target Cells to HIV and Inhibitors. H9 and U937 cells grown in RPM1 1640 medium containing 20% (vol/vol) fetal calf serum (R20 medium) (13) were resuspended in serum-free Eagle's minimal essential medium (MEM) in presence and absence of inhibitors at 37°C for 30 min or less. HIV, 10^6 to 10^7 $TCID_{50}$ units per 10^6 cells, was then added for 2 h. Inhibitors and unbound virus were removed by centrifugation and the cells were resuspended in R20 medium and grown in 24-well plates (8×10^5 cells per well) at 37°C for 7 days (H9) and 14 days (U937). The period of virus–cell interaction in presence of inhibitors was kept short to specifically test inhibitor interference with virus entry. Serum-free MEM was used during exposure as in experiments on inhibition of DT cytotoxicity (2, 3). Control experiments were performed in which exposure was carried out in R20 medium. Under these conditions, virus infection was 10% of that in serum-free MEM (10.4 ± 7%), but the percent inhibitions caused by 2.5 mM DTNB and 3.0 mM bacitracin were high (79 ± 14% and 73 ± 7%, respectively) and increased to 98 ± 1% when the concentrations of inhibitors were doubled to compensate for their interaction with serum components. Peripheral blood mononuclear cells (PBMCs) were isolated from control subjects, resuspended to $1–2 \times 10^6$ cells per ml in R20 medium, and cultured at 37°C in 5% CO_2/95% air with 1% (by volume) phytohemagglutinin

Abbreviations: PDI, protein disulfide-isomerase; DT, diphtheria toxin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PBMC, peripheral human mononuclear cell; HIV, human immunodeficiency virus; DTT, dithiothreitol; mAb, monoclonal antibody.

†To whom reprint requests should be addressed.

type M (Difco) for 3 days. They were exposed to drugs and/or HIV (2–5 × 10^6 $TCID_{50}$ units per 4 × 10^6 cells) in serum-free medium and, after removal of inhibitors and unbound virus, were resuspended to 1 × 10^6 cells per ml in R20 medium with 5% (by volume) interleukin 2-containing medium supplement (Human T-stim, Collaborative Research). The cells were harvested on day 7 or 10. When anti-PDI mAbs were used as inhibitors, the serum-free medium of control cells contained equivalent amounts of irrelevant mouse IgG. Exposures of target cells to inhibitors alone were monitored for possible cytotoxicity or inhibition of cell proliferation by using the MTT assay, whose sensitivity is comparable to that of measuring [^{3}H]thymidine incorporation into DNA (14). Cells exposed to inhibitors alone were also tested for their ability to bind anti-CD4 antibodies. They were resuspended in phosphate-buffered saline (with 1% fetal bovine serum) containing fluorescein-labeled CD4 (Leu-3a) for a 30-min incubation on ice and fixed in 1% paraformaldehyde. The CD4-specific fluorescence was analyzed in a FACScan flow cytometer (Becton Dickinson), using the FACSCAN and LYSYS programs (Becton Dickinson).

Measurement of HIV Infection. At the end of the growth period, cell-free culture supernatants containing 0.5% Triton X-100 were assayed for p24 viral protein by using the HIV-1 p24 core profile ELISA (DuPont/NEN). The KINETICALC program from Bio-Tek (Burlington, VT) was used for kinetic and end-point analyses of p24. Supernatants of several experiments were also assayed for infectious virus using C8166 cells as targets and syncytium formation as the end point (15).

RESULTS

Inhibitory Effect of Membrane-Impermeant Thiol Reagents. When H9 cells were exposed to inhibitor and virus, 2.5 mM DTNB decreased p24 release to <1% of control (Table 1). The titer of infectious virus determined in the supernatant of one experiment was decreased by five orders of magnitude compared to controls. When exposure to DTNB prior to infection was reduced from 30 min to 1 min, the level of inhibition remained comparable (93 ± 2% vs. 99.9 ± 0.1%). When the culture of infected H9 cells was reduced from 7 to 4 days, the relative inhibitions were unchanged, even though the absolute values of p24 released by control cells were 18-fold lower (Fig. 1). The DTNB-induced inhibition was dose-dependent. The IC_{50} of 0.3 mM taken from Fig. 1 was close to the IC_{50} of 0.1 mM obtained for DTNB-induced inhibition of soluble PDI activity (3), suggesting that the inhibitory effect of DTNB reported in Table 1 and Fig. 1 was due to an inhibition of PDI. The membrane-impermeant sulfhydryl reagent Thiolyte MQ tested at 0.3 and 1.0 mM inhibited infection by 44.3 ± 34% and 88%, respectively, suggesting a dose–effect relationship similar to that of DTNB. Inhibitions of HIV infection similar to those recorded with H9 cells were seen with macrophage-derived U937 cells and human PBMCs (Table 2).

Table 1. Percent inhibition of HIV infection by three classes of inhibitors in H9 cells

Inhibitor	% inhibition		
	Average p24 level	p24 level	Infectious titer
DTNB (2.5 mM)	99.9 ± 0.12 (4)	99.9	99.9
Bacitracin (3.0 mM)	88 ± 12 (5)	96.9	99.9
mAb			
HP13 (220 μg/ml)	83 ± 3.4 (3)	79.5	90.9
RL90 (290 μg/ml)	90 ± 4.4 (3)	—	—

For the average p24 level, percent inhibitions were calculated for each experiment and shown as the average ± SD. Absolute p24 values for controls ranged from 5.3 to 570 ng/ml (average 9.8). The number of experiments is in parentheses. p24 levels and infectious titers were measured in quadruplicate supernatants of the same experiment. Titers for controls were 1.7 × 10^7, 1 × 10^7, and 5.8 × 10^5 $TCID_{50}$ units/ml in experiments testing DTNB, bacitracin, and HP13, respectively. These inhibitors decreased the titers by factors of 2.1 × 10^5, 710, and 11, respectively. Antibodies were partially purified from conditioned medium by protein G column chromatography.

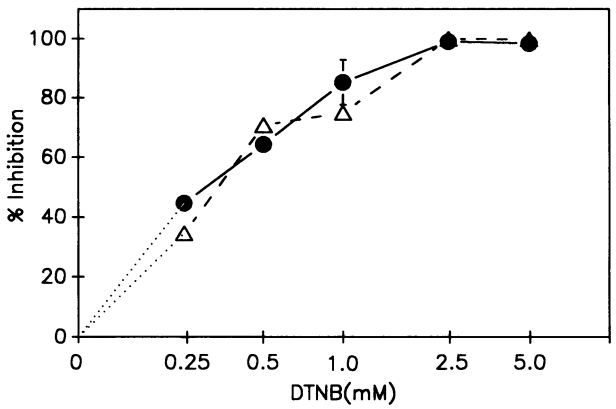

FIG. 1. Dose dependence of the DTNB-induced inhibition of HIV infection. H9 cells (8 × 10^5 cells per ml) were exposed to DTNB, and the growth period at 37°C was either 4 (△) or 7 days (●). The points are averages of triplicates in one (△) or two (●) experiments; the 1.0 mM DTNB value at 7 days is the average ± SD of five experiments. The absolute amount of p24 in supernatants of controls at 4 and 7 days was 0.035 and 0.62 ng/ml, respectively.

In view of the prior finding that DTNB inhibits the reductive cleavage of surface-bound disulfide conjugates (1) and the reductive activation of membrane-bound DT (2), it is reasonable to hypothesize that it similarly blocks the cleavage of critical disulfide bonds in membrane-bound HIV and that such cleavage is required for HIV infection. To test whether HIV contains cleavable disulfide bonds, HIV was pretreated at 37°C with the reducing agent dithiothreitol (DTT), and its infectivity toward C8166 was determined. Exposure to DTT decreased HIV infectivity in a dose-dependent fashion by about three orders of magnitude (Fig. 2). These data indicate that disulfide bonds of HIV are accessible to DTT and are necessary for HIV infection. While this DTT-induced loss of infectivity is mostly due to conformational changes that decrease virus binding, the finding does not preclude that, in intact cells, an event after binding involving a disulfide cleavage is essential for infection. The inhibitions of HIV infection shown in Table 1 and Fig. 1 could, however, also be ascribed to (*i*) an inhibitory effect of DTNB on HIV infectivity prior to binding, (*ii*) a DTNB-induced decrease of HIV binding to its receptor, or (*iii*) a DTNB-induced inhibition of the growth of target cells or their capacity to support viral replication. These three possibilities were ruled out as follows.

Table 2. Inhibition of p24 release by DTNB and bacitracin in three human cell types

Cell type	Inhibition of p24 release, %	
	DTNB	Bacitracin
H9	99.9 ± 0.1 (4)	88 ± 12 (4)
U937	88 ± 10 (6)	44 ± 24 (5)
PBMC	77 ± 28 (6)	91 ± 10 (6)

Inhibitions were calculated as in Table 1. The absolute values of p24 in controls ranged from 5.3 to 140 ng/ml (average, 68), 0.023 to 1.2 (average, 0.46), and 0.04 to 57 (average, 18), for H9 cells, U937 cells, and PBMCs, respectively. The number of experiments is given in parentheses.

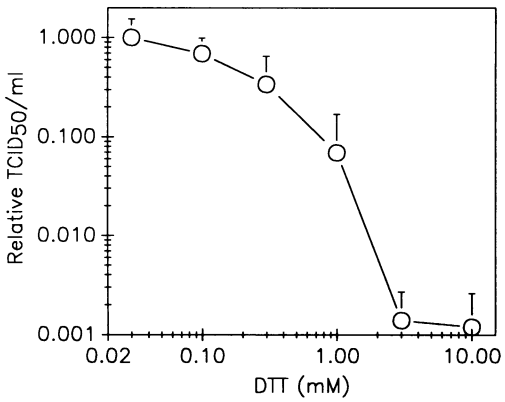

FIG. 2. Decrease in HIV infectivity caused by DTT. HIV of very high titer (5×10^{12} $TCID_{50}$ units/ml) was exposed for 2.5 h at 37°C to increasing concentrations of DTT in serum-free MEM. The virus/DTT solution was serially diluted with MEM and virus aliquots were added to wells containing C8166 cells in R20 growth medium, inoculating six wells for each dilution and growing the cells for 7 days at 37°C. The titers were determined by the virus dilutions at which heterokaryons were detected in three out of six wells ($TCID_{50}$ units/ml) (15). Dilution brought the residual concentration of DTT to a level known to be nontoxic to C8166 cells in all determining groups of six wells. The decreases of $TCID_{50}$ relative to untreated virus were calculated in each experiment and averaged. The absolute titer of controls was 3.9×10^8 $TCID_{50}$ units/ml ($n = 9$). Points are averages of three to eight experiments.

(*i*) HIV at a titer 10^5 times higher than that used for infection was exposed to 5.0 mM DTNB in serum-free medium for 2.5 h at 37°C and the virus/DTNB mixture was diluted $1:10^5$, which brought DTNB to a concentration known to have no effect on HIV infection (Fig. 1) or on disulfide reduction (1–3). This treatment had no significant effect on the infection of H9 cells as measured by p24 release (73.5 ± 31% of controls, $n = 3$). Treated virus, thus, was capable of binding to its receptor and of infecting cells. (*ii*) The possibility that DTNB might decrease virus binding by down-regulating or impairing the integrity of CD4 receptors on target cells was tested by measuring the binding of fluorescein-labeled anti-CD4 antibody by cytofluorimetry. Labeling was indistinguishable in control and treated cells in all five experiments performed. In a typical experiment, the mean channel values for CD4-specific fluorescence intensities were 115.2 ± 15.5 and 113.2 ± 15.4 for controls and DTNB-treated cells, respectively. A similar lack of effect of DTNB was observed in four experiments on PBMCs, in which the peaks of CD4-positive and -negative cells were separated sharply. In a typical experiment, in which 50% of the cells were CD4-positive, the mean channel fluorescence intensities for CD4-positive cells were 99.5 ± 11.1 and 101.4 ± 10.5 for control and DTNB-treated cells, respectively. Similar results were obtained when either cell line was treated with 1.0 mM Thiolyte MQ or 3.0 mM bacitracin. Since DTNB impaired neither the capacity of pretreated virus to bind to its receptor nor the capacity of CD4 to bind a specific anti-CD4 antibody, it is reasonable to infer that it does not hinder virus–receptor interaction, especially since similar hindrances would have to be caused by inhibitors as dissimilar in structure as DTNB, Thiolyte MQ, and bacitracin.

(*iii*) The possibility that DTNB might decrease infection by decreasing the number or metabolism of target cells was tested using the MTT cytoxicity assay (14). H9 cells exposed to inhibitor alone were tested after 1, 2, and 6 days of culture. The MTT values were 81 ± 29%, 91 ± 9%, and 100% of controls at days 1, 2, and 6, respectively. U937 cells and PBMCs were similarly unaffected. The possibility that even a short exposure to DTNB might have a protracted inhibitory effect on virus replication was tested by exposing H9 cells first to HIV to initiate infection and then to DTNB before transfer to regular growth medium. This treatment did not suppress virus infection, as indicated by a released p24 antigen level of 92.2 ± 13.5% of controls. Exposure of chronically HIV-infected H9 cells to DTNB did not influence the virus replication during the subsequent 7 days of growth, as indicated by a released p24 level of 91 ± 26% of controls.

These control experiments indicate that to cause the inhibitions reported in Table 1 and Fig. 1, DTNB must act at the time of initial virus–cell interaction and support the interpretation that, by blocking cell surface sulfhydryls, DTNB prevents cleavage of viral disulfides required for the normal sequence of HIV infection. The finding that both intact surface sulfhydryls and intact viral disulfide bonds are required for an infective HIV–cell interaction is consistent with the view that the two engage in a thiol–disulfide interchange.

Inhibitory Effect of Anti-PDI Antibodies. mAb HP13 directed against human PDI (12) inhibited HIV infection of H9 cells by 83 ± 3.4% (Table 1). This inhibition was greater than expected, since the effect of HP13 on PDI activity was found to level off at 50% inhibition even at a 10- to 15-fold molar excess of mAb over antigen (12). This upper limit suggests, among other possibilities (12), that the mAb might bind to only one of the two active sites of soluble PDI (16). Exceeding that limit might in turn suggest that only one active site is exposed at the surface of target cells—namely, the site that binds HP13. Despite the limited *in vitro* effect of HP13 on soluble PDI, a correlation between its effect on PDI activity and HIV infection was seen. A HP13 preparation purified from ascites fluid that caused only 17% inhibition of PDI activity at 100 μg/ml also caused a lesser inhibition of HIV infection (32% at 290 μg/ml). In the experiment of Table 1, in which release of p24 antigen and infectious virus were both measured in the same supernatant, the inhibitions were 79.5% and 90.9% of control, respectively. The mAb RL90 directed against rat PDI (12), which had previously been found to be more effective than HP13 in inhibiting DT cytotoxicity (3) and the activity of human PDI (12), caused a 90 ± 4.4% inhibition of p24 release (Table 1). These data confirm that RL90 cross-reacts with human PDI (12), as it does with hamster PDI (3). Preliminary screening of H9, U937, and C8166 cells for surface-associated PDI by FACScan analysis using anti-PDI mAbs combined with fluorescein isothiocyanate-conjugated $F(ab')_2$ anti-mouse IgG revealed immunoreactive PDI at the surface of all three target cells. PDI-specific fluorescence intensities were greater with RL90 than with HP13.

Inhibitory Effect of Bacitracin. The antibiotic bacitracin, known to inhibit both the reductive (17) and the oxidative (18) functions of PDI, inhibited HIV infection in H9 cells by 88 ± 12% at 3.0 mM (Table 1). That concentration inhibits the activity of soluble PDI by 95% and the cytotoxicity of DT by 87% (3). In the experiment of Table 1 in which p24 release was decreased by 96.9%, the titer of infectious HIV in the same supernatant was decreased by 99.9%. Bacitracin also markedly decreased HIV infection in U937 cells and PBMCs (Table 2). Control experiments showed that bacitracin did not inactivate HIV prior to binding, did not decrease the binding capacity of CD4, and did not decrease the proliferative capacity of infected cells. Bacitracin is transported poorly into mammalian cells and is capable of inhibiting the reductive cleavage of a membrane-bound disulfide conjugate at 0°C (3), a temperature that precludes endocytosis of the conjugate. It can be assumed, therefore, that bacitracin, like DTNB and anti-PDI mAbs, inhibits PDI at the surface of target cells. Bacitracin has been found to inhibit several bacterial proteases, neuropeptidases, leucylpeptidase, insulinase, transglutaminase, and papain, some of which have cysteine residues in their active sites. No plausible link can

be established at this time between these inhibitory effects and those reported in Tables 1 and 2. The comparison of bacitracin, anti-PDI antibodies, and DTNB (Table 1) shows that DTNB causes the strongest inhibitions of infection, presumably, because it blocks all four critical thiols of PDI (16) and, perhaps, other membrane thiols that may be required to regenerate oxidized PDI.

DISCUSSION

Our data show that the membrane-impermeant thiol reagent DTNB, which abolishes the reductive cleavage of a surface-bound disulfide conjugate (1) and the reductive activation of receptor-bound DT in CHO cells (2), is also capable of inhibiting infection of human lymphoid cells by membrane-bound HIV. Our data also show that two inhibitors of PDI, bacitracin and anti-PDI mAbs, which inhibit the same two reductive processes (3), similarly inhibit HIV infection. At 1.0 mM, DTNB causes 100% inhibition of soluble PDI activity (3). It can be assumed, therefore, that the critical sulfhydryls blocked by DTNB include the two cysteine thiols present at both active sites of PDI (16). PDI catalyzes thiol–disulfide interchanges leading to the correct folding of nascent proteins in the endoplasmic reticulum (4). Yet there is both immunoelectronmicroscopic evidence (5, 6) and functional evidence (3) that PDI is also present at the surface of mammalian cells. We had previously reported that the cleavage of disulfide bonds in membrane-bound ligands resulted from interchanges with the thiols of membrane-associated PDI and that, for DT activation, the interchange occurred upon ligand–receptor interaction (3). We now suggest that similar interchanges occur between PDI and disulfides in envelope proteins of membrane-bound HIV at the time of virus–receptor interaction.

This of course implies that membrane thiols interact with disulfides present in the receptor binding domain of gp120. Leonard *et al.* (10) reported that gp120 contains nine conserved disulfide bonds, three of which are situated in the vicinity of the CD4 binding domain (10, 11). The position of these three disulfide bonds and their relationship to CD4 binding regions of gp120 are represented in Fig. 3. The disulfide linkages between residues 378 and 445 and residues 385 and 418 are close to binding sites in C3 and C4. The disulfide bond between residues 296 and 331 forms the V3 loop. Recent studies suggest that a close spatial relationship exists between V3 and the primary binding site located in C4 (19, 20) and that protein folding may bring the base of the V3 loop close to that site (19). A recent investigation of the interaction of PDI with peptides has identified a 26-residue peptide binding site close to the C-terminal of PDI with an extremely high content of acidic amino acids (21). This anionic sequence might favor an interaction of PDI with cationic regions of gp120, in particular the V3 loop, whose basic character is well known (22). The 34 residues of the V3 loop of HIV-1 (Fig. 3) include 6 Arg and 2 Lys residues. The consensus sequence of V3 is somewhat less basic (4 Arg and 1 Lys residues). Our analysis of two recent studies on the heterogeneity of V3 in Brazilian (23) and Parisian (24) individuals revealed that the V3 loop averages 5.2 ± 1.0 basic residues in a combined pool of 62 HIV-1 isolates. Another pool of 44 HIV-1 samples with different V3 sequences from U.S.A. patients (22) averaged 6.0 ± 1.2 basic amino acid residues. The conserved C3 and C4 regions of gp120 depicted in Fig. 3 also have a relatively high content of basic amino acids (5 Arg and 7 Lys residues). It thus appears that all three disulfide bonds shown in Fig. 3 are potential substrates for a thiol–disulfide interchange after HIV–CD4 interaction. Evidence that these three disulfides are required for infection is provided by site-directed mutation experiments, in which replacement of one or both cysteine residues at any of the

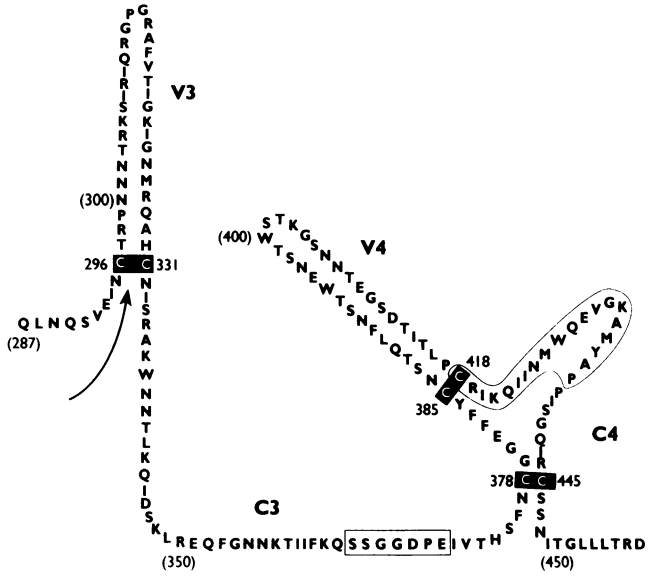

FIG. 3. Position of disulfide bonds in relation to the CD4 binding domains of gp120. Partial sequence of gp120 of HIV-1, isolate BH10, taken from the National Center for Biotechnology Information, National Library of Medicine data base. The disulfide bonds forming the V3 and V4 loops or linking amino acid residues 378 and 445 are represented (C-C). The two surrounded sequences are CD4 binding regions, according to Larsen *et al.* (11). Recent studies suggest a close spatial relationship between the base of the V3 loop and the primary binding site in C4 (19). The arrow points to the site where a fusogenic gap might be created by a joint action of PDI and a V3-specific protease.

three sites results in noninfectious mutants (25). Interestingly, two mutants in which the Cys–Cys pairs linking residues 378–445 or 385–418 are replaced by Ser/Ser or Val/Ser bind normally to CD4 (36), suggesting that disulfide bonds at these positions may be important in events occurring after binding. A fourth disulfide bond linking residues 119 and 205 and forming the V1/V2 disulfide domain (10) is adjacent to a region of C1 that deletion data indicate is important for CD4 binding (26). It may also be a substrate for surface-associated PDI.

It is generally thought that HIV binding to CD4 causes conformational changes in envelope proteins that ultimately allow the virion envelope to fuse with the cell membrane (11, 27, 28). We speculate that an early reaction that triggers this sequence of events is the PDI-catalyzed reduction of disulfide bonds in gp120. Marked conformational changes would follow the cleavage of any of the four above-mentioned disulfide bonds. Conformational changes in Sindbis virus envelope proteins after virus attachment have been detected by the occurrence of new transient epitopes (29) and similar changes have been elicited by mild treatment of the virus with DTT (30). At higher concentrations, DTT abolishes Sindbis virus infectivity (7), in analogy to the loss of HIV infectivity seen in Fig. 2. It was shown also that Sindbis infection of BHK cells is inhibited by DTNB at concentrations that do not alter virus infectivity (8, 9). It would appear, therefore, that reduction of disulfide bonds might be a key event in both Sindbis and HIV infection. In both cases the possibility must be considered that virus–receptor interaction might itself cause conformational changes that render disulfide bonds more accessible or susceptible to thiol–disulfide interchange.

Summarizing the above discussion, the postulate that thiol–disulfide interchange between PDI thiols and gp120 disulfides plays a key role in triggering virus entry is based on the following lines of reasoning. (*i*) Three classes of agents that inhibit the activity of soluble PDI also inhibit HIV infection at comparable concentrations. (*ii*) Three disulfide

bonds situated in CD4 binding domains of gp120 are plausible substrates for surface-associated PDI present in the vicinity of the CD4 receptor. Cleavage of any of these bonds would cause important conformational changes potentially capable of triggering virion–cell fusion. (*iii*) Evidence that surface-associated PDI can act on a receptor-bound ligand is provided by the activation of DT and its inhibition by PDI inhibitors (3). Although biochemical evidence for the postulated PDI-catalyzed thiol–disulfide interchange is not yet available, isomerization provides a biochemical strategy. A PDI-induced interchange would indeed form a new, albeit transient, disulfide bond between PDI and a cysteine residue of gp120. This should make it feasible to identify a PDI adduct. Two other cellular oxidoreductases capable of cleaving intracellular protein disulfide bonds, namely, glutaredoxin and thioredoxin, are improbable participants in surface-associated reductive cleavages, since neither is likely to cross-react with anti-PDI mAbs and since thioredoxin, which has the same dithiol motif as PDI in its active site, is not inhibited by bacitracin (3).

The postulated functional association of CD4 and PDI resulting in the reduction of disulfide bonds in gp120 introduces a requisite for HIV infection and suggests that HIV binding to a CD4 receptor that is not spatially associated with PDI would not be sufficient for infection. The requirement of a second factor acting in concert with CD4 binding has been postulated by several groups of investigators on the ground that certain cells expressing or made to express CD4 are capable of binding HIV with high affinity and yet are resistant to HIV (see refs. 11 and 28). Such cases could be explained by the absence or inappropriate positioning of PDI in the receptor area of resistant cells. Other examples suggestive of cooperativity between receptor binding and enzymatic cleavage have been seen in systems where the interaction of gp120 with soluble CD4 increases the susceptibility of V3 to proteolytic nicking by thrombin (31, 32) or by some endogenous protease (33). A proteolytic attack on V3 combined with a disulfide cleavage opening the base of the V3 loop would be particularly meaningful (as can be inferred from Fig. 3), since it would transform V3 into a channel through which the viral envelope might reach and fuse with the target-cell membrane. This model would apply to the joint action of PDI with any endogenous V3-specific protease, regardless of its cleavage site, including for instance the serine esterase TL2 (34), cathepsin E-like and thrombin-like proteinases (31), and dipeptidyl peptidase type IV (35). The model also may help explain the fusogenic properties of the V3 loop postulated by a number of investigators (11, 27, 28, 31, 32). It must be considered, however, that cleavage of disulfide bonds may trigger key molecular events in other domains of gp120 and possibly in gp41, whose lone disulfide bond (Cys-598–Cys-604) is in a region that becomes exposed after binding of sCD4 to gp120 on infected cells (ref. 32 and Q. J. Sattentau, personal communication). Cleavages at such sites may also provide insights into the process of HIV–cell fusion.

The suggested interaction of surface-associated PDI with viral disulfides may offer other approaches to the design of anti-HIV drugs (namely, the development of inhibitors of surface-associated PDI) and the development of neutralizing antibodies targeted specifically to epitopes containing viral disulfide bonds in the conserved CD4 binding regions of gp120. More generally, these findings may assign significance to disulfide bonds present in the outer proteins of other viruses or other microorganisms, by suggesting that they may play a role in the triggering of infection.

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1. Feener, E. P., Shen, W.-C. & Ryser, H. J.-P. (1990) *J. Biol. Chem.* **265**, 18780–18785.
2. Ryser, H. J.-P., Mandel, R. & Ghani, F. (1991) *J. Biol. Chem.* **266**, 18439–18442.
3. Mandel, R., Ryser, H. J.-P., Ghani, F., Wu, M. & Peak, D. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 4112–4116.
4. Freedman, R. B. (1989) *Cell* **57**, 1069–1072.
5. Varandani, P. T., Raveed, D. & Nafz, M. A. (1978) *Biochim. Biophys. Acta* **538**, 343–353.
6. Yoshimori, T., Semba, T., Takemoto, H., Akagi, S., Yamamoto, A. & Tashiro, Y. (1990) *J. Biol. Chem.* **265**, 15984–15990.
7. Anthony, R. P., Paredes, A. M. & Brown, D. T. (1992) *Virology* **190**, 330–336.
8. Brown, D. T. & Edwards, J. (1992) *Semin. Virol.* **3**, 519–527.
9. Abell, B. A. & Brown, D. T. (1993) *J. Virol.* **67**, 5496–5501.
10. Leonard, C. K., Spellman, M. W., Riddle, L., Harris, R. J., Thomas, J. N. & Gregory, T. J. (1990) *J. Biol. Chem.* **265**, 10373–10382.
11. Larsen, C., Ellens, H. & Bentz, J. (1992) in *Membrane Interactions of HIV*, eds. Aloia, R. C. & Curtin, C. C. (Wiley–Liss, New York), pp. 143–166.
12. Kaetzel, C. S., Rao, C. K. & Lamm, M. E. (1987) *Biochem. J.* **241**, 39–47.
13. Johnson, V. A., Barlow, M. A., Merrill, D. P., Chou, T.-C. & Hirsch, M. S. (1990) *J. Infect. Dis.* **161**, 1059–1067.
14. Mosmann, T. J. (1983) *Immunol. Methods.* **22**, 1759–1770.
15. Hartshorn, K. L., Vogt, M. W., Chou, T.-C., Blumberg, R. S., Byington, R., Schooley, R. T. & Hirsch, M. (1987) *Antimicrob. Agents Chemother.* **31**, 168–172.
16. Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A. & Rutter, W. J. (1985) *Nature (London)* **317**, 267–270.
17. Roth, R. A. (1981) *Biochem. Biophys. Res. Commun.* **98**, 431–438.
18. Mizunaga, T., Katakura, Y., Miura, T. & Maruyama, Y. (1990) *J. Biochem. (Tokyo)* **108**, 846–851.
19. Moore, J. P., Thali, M., Jameson, B. A., Vignaux, F., Lewis, G. K., Poon, S.-W., Charles, M. A., Fung, M. S., Sun, B., Durda, P. J., Åkerblom, L., Wahren, B., Ho, D., Sattentau, Q. J. & Sodroski, J. (1993) *J. Virol.* **67**, 4785–4796.
20. Wyatt, R., Thali, M., Tilley, S., Pinter, A., Posner, M., Ho, D., Robinson, J. & Sodroski, J. (1992) *J. Virol.* **66**, 6997–7004.
21. Noiva, R., Freedman, R. B. & Lennarz, W. J. (1993) *J. Biol. Chem.* **268**, 19210–19217.
22. Milich, L., Margolin, B. & Swanstrom, R. (1993) *J. Virol.* **67**, 5623–5634.
23. Potts, K. E., Kalish, M. L., Lott, T., Orloff, G., Luo, C.-C., Bernard, M.-A., Brites Alues, C., Badaro, R., Suleiman, J., Ferreira, D., Schocherman, G., Johnson, W. D., Jr., Ou, C.-Y. & Ho, J. L. (1993) *AIDS* **7**, 1191–1197.
24. Chaix, M.-L., Cappey, C., Couillin, I., Rozenbaum, W., Levy, J.-P. & Saragosti, S. (1993) *AIDS* **7**, 1199–1204.
25. Tschachler, E., Bucho, H., Gallo, R. C. & Reits, M. S., Jr. (1990) *J. Virol.* **64**, 2250–2259.
26. Syu, W. J., Huang, J.-H., Essex, M. & Lee, T.-H. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3695–3699.
27. Eiden, L. E. & Lifson, J. D. (1992) *Immunol. Today* **13**, 201–207.
28. Capon, D. J. & Ward, R. H. R. (1991) *Annu. Rev. Immunol.* **9**, 649–678.
29. Flynn, D. C., Meyer, W. J., MacKenzie, J. M., Jr., & Johnston, R. E. (1990) *J. Virol.* **64**, 3643–3653.
30. Meyer, W. J., Gidwitz, S., Ayers, V. K., Schoepp, R. J. & Johnston, R. E. (1992) *J. Virol.* **66**, 3504–3513.
31. Clements, G. J., Price-Jones, M. J., Stephens, P. E., Sutton, C., Schutz, T. F., Clapham, P. R., McKeating, J. A., McLure, M. O., Thomson, S., Marsh, M., Kay, J., Weiss, R. A. & Moore, J. P. (1991) *AIDS Res. Hum. Retroviruses* **7**, 3–16.
32. Sattentau, Q. J. & Moore, J. P. (1991) *J. Exp. Med.* **174**, 407–415.
33. Werner, A. & Levy, J. A. (1993) *J. Virol.* **67**, 2566–2574.
34. Kido, H., Fukutomi, A. & Katunuma, N. (1990) *J. Biol. Chem.* **265**, 21979–21985.
35. Callebaut, C., Krust, B., Jacotot, E. & Hovanessian, A. G. (1993) *Science* **262**, 2045–2050.
36. Lekutis, C., Olshersky, U., Furman, C., Thali, M. & Sodroski, J. (1992) *AIDS* **5**, 78–81.