Inhibitors of Protein-Disulfide Isomerase Prevent Cleavage of Disulfide Bonds in Receptor-bound Glycoprotein 120 and Prevent HIV-1 Entry*

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The entry of human immunodeficiency virus type 1 (HIV-1) into target cells requires three successive virus-cell interactions, namely the binding of the HIV-1 envelope glycoprotein gp120 (Env) to the primary receptor CD4, the attachment of CD4-bound gp120 to coreceptors CCR5 or CXCR4 (1), and ultimately, the fusogenic activation of envelope glycoprotein gp41 (2–4). It is generally accepted that virus binding to CD4 causes major conformational changes in gp120 (5, 6), but the molecular mechanisms triggering these changes have not been fully characterized. Immunologically detectable conformational changes in gp120 occur upon its binding to the soluble ectodomain of CD4 (sCD4). They expose new gp120 epitopes, one of which overlaps with the acid pH-induced conformational changes, which drive the entry of influenza virus, have been identified in HIV-1 entry. Here we describe how a known membrane-associated oxidoreductase, protein-disulfide isomerase (PDI, EC 5.3.4.2), may cause the major structural changes in HIV-1 envelope that lead to virus entry.

PDI is a well characterized 57-kDa oxidoreductase (9) that forms, breaks, and rearranges disulfide bonds in nascent proteins reaching the endoplasmic reticulum (10). Its redox function is based on the presence in its two active sites of a Cys-Gly-His-Cys (CXXC) motif. When the cysteines of CXXC are oxidized, PDI interacts with two cysteiny1 thiol groups of a neighboring peptide to form a disulfide bond. When CXXC bears two cysteiny1 thiois, it cleaves neighboring disulfide bonds. In the endoplasmic reticulum, PDI acts predominantly as an oxidase (10–12). At the cell surface, however, PDI acts as a reductase (13–21), and one of its functions is to catalyze thiol-disulfide interchanges. An interchange generates two free thiols in a target protein and an oxidized CXXC motif (22). PDI is inherently a promiscuous enzyme. Its property to bind to different peptides is essential to its function both inside the cell and at the cell surface. The surface functions of PDI are many and include interacting with the receptor of diphtheria toxin to facilitate the transport of the toxic fragment into the cytoplasm (13, 14), reducing a disulfide bond in the ectodomain of the thyrotropin receptor (17), binding to extracellular thrombospondin (18), and binding nitric oxide in a transnitrosation reaction required for the transport of nitric oxide into megakaryocytes and for the maturation of platelets (19). PDI may reach the surface in association with other endoplasmic reticulum proteins transported to the cell membrane through a pathway that is subject to regulation (54). PDI is shed from the...
was vacuum. The purity of AT3 determined by thin-layer chromatography 3 times with 20 ml of water and dried at room temperature under 10 M HCl and kept for 30 min at 4 °C. After washing, the gel was dried at 30 °C. The excised bands were washed with ice-cold lysis buffer, and proteins eluted with 0.5 M NaCl, 150 mM NaCl, and 10 mM Tris and transferred. The membrane was probed for PDI with mAb R77, and reprobed for CD4 with CD4 anti-serum. Cellular CD4 and PDI were also immunoprecipitated with anti-CD4 mAb Leu3a and, upon probing with HRP-NeuAvidin, was not biotinylated.

Coprorecipitation of Soluble CD4 by Affinity-labeled Soluble PDI—Soluble PDI biotinylated on its thiol with membrane-impermeant MPMDO was purified by ultrafiltration and mixed with an equimolar amount of non-biotinylated soluble CD4 (1 h at 25 °C). After precipitation with immobilized avidin, separation by SDS-PAGE, and transfer to nitrocellulose membrane, PDI and CD4 were immunodetected on the same blot with mAbs T77 and CD4 antiserum. To assess the reductive activity of the cell surface, labeled [125I]tyramine-SS-poly(-lysine) was bound to cells at 0 °C for 1 h. The cells were then washed and incubated in PBS (30 min at 37 °C). After PDI had been inactivated by 80 mM N-ethylmaleimide, cells were lysed with 0.5% Triton X-100, and the 25% trichloroacetic acid-soluble radioactivity released during incubation was determined.

Coprorecipitation of Soluble CD4 by Biotinylated Soluble PDI—Soluble PDI biotinylated on its thiol with membrane-impermeant MPMDO was added in a 1:1 molar ratio to the CD4 preparation. The mixture was incubated on ice for 1 h. After addition of 200 μg/ml leupeptin, 20 μg/ml of type II collagenase and 1% Brij-35, the mixture was centrifuged at 10,000 × g for 15 min. The supernatant was centrifuged again at 120,000 × g for 1 h and the supernatant was used for the coprecipitation experiments. The mixture was then incubated on ice for 30 min. The precipitated CD4 and PDI were then washed 5 times with ice-cold saline solution and proteins eluted with Laemli buffer containing 50 mM dithiothreitol. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and duplicate blots were probed for CD4 with mAb SIM-2 or for PDI with mAb RL77.

Coprorecipitation by Surface Cells (Biotinylated PDI on gp120)—The surface of 5 × 10^5 U937 cells was labeled with 1 ml of 0.5 mM membrane-impermeant thiol-specific MPMDO (0 °C for 30 min) to tag the thiol of PDI and other surface proteins. Cells were washed with cold Hanks’ buffered saline solution and lysed in 200 μM Tris, pH 8.3, containing 0.6% CHAPS, 50 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml aprotinin. Biotinylated proteins were isolated with immobilized avidin and resolved by SDS-PAGE and transferred. The membrane was probed for PDI with mAb R77, stripped, and reprobed for CD4 with anti-CD4 serum. Cellular CD4 and PDI have a similar electrophoretic mobility. This probing identified both proteins.

Coprorecipitation of gp120-Dissulfide Bonds by Soluble PDI—Recombinant gp120<sub>16</sub> (100 μM) was treated with thrombin (200 μM, 120 min at 37 °C) to make a proteolytic cut near the tip of the V3 loop. Subsequent reduction of the V3-dissulfide bond is known to cleave gp120 into a 70-kDa N-terminal and a 50-kDa C-terminal fragment (33). Reduction was carried out with 5% 2-mercaptoethanol (control) or with PDI (50 μg/ml, 1 h at 37 °C). Following separation by SDS-PAGE under non-reducing conditions, gp120 and its fragments were detected with polyclonal anti-gp140 antibody R2143.

Detection of Generated Thiols in gp120 (gp120<sub>16</sub> Bound to Target Cells)—Recombinant gp120<sub>16</sub> (100 μM) or oligomeric gp140<sub>16</sub> (10 μg/ml) (34) were allowed to bind to 5 × 10<sup>5</sup> target cells (1 h at 14 °C) in Hanks’ buffered saline solution without PDI inhibitors. After washing, the cells were labeled (40 min at 0 °C) with

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EXPERIMENTAL PROCEDURES

Reagents—PDI was isolated from calf liver (13) or purchased from Pierce. The thiol-specific biotinylating agents and biotin-specific agents used were as follows: membrane-impermeant biotinyl 3-maleimido propionamidyl-3'-dioxoacetic acid (MPDO, Molecular Biosciences, Biotech, OH); Biotin-BMCC, Ultralink Immobilized NeutrAvidin, and HRP-NeutrAvidin (all from Pierce); phenylarsine oxide (PAO), T3, and DTNB; and thrombin and anti-CD4-FITC mAb Q4120 (Sigma). Para-acetylated T3 (AT3). After centrifugation, the precipitate was washed 5 times with ice-cold saline solution and proteins eluted with Laemli buffer containing 50 mM dithiothreitol. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and duplicate blots were probed for CD4 with mAb SIM-2 or for PDI with mAb RL77.

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Biotin-BMCC (0.5 mM) to biotinylate newly generated thiol, washed, and lysed (20 min at 4 °C) in PBS containing 0.25% Nonidet P-40 (or 0.6% CHAPS), 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 20 μM phenylmethylsulfonyl fluoride. When using gp140, the envelope glycoprotein was immunoprecipitated with rabbit polyclonal anti-gp140 antibody R2143 and immobilized protein G, resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed for biotin using HRP-NeutAviDin. Western blots were stripped and reprobed with mouse anti-gp120 mAb D19 to assess Env binding to CD4. When using gp120mut, thiol-biotinylated surface proteins were isolated with immo-
obilized avidin. Western blots were then probed for Env with Ab R2143 and probed for PDI with mAb RL77. They were stripped and reprobed for CD4 with CD4 antisera. In other experiments, the CCR5-tropic gp120mut was co-purified with U87 CD4 cells that did or did not express the CCR5 coreceptor. The newly generated protein thiol was biotinylated, isolated, and reprobed for envelope as in experiments using gp120mut.

Virus Production—HIV-1NL4-3 and HIV-1JR-FL viruses were generated by infection of SupT1 cells and FM1 cells, respectively, with cell-free viruses. Alternatively, SupT1 cells were transfected with plNL4-3. The pseudotyped HIV-1 was generated by transfection of COS-7 with pSV-
A-MLV-Env and with pHIV-gpt (15 μg each) according to Page et al. (35). HIV-1 titers were measured using an HIV-1 p24 antigen capture enzyme-linked immunosorbent assay (Coulter Immunotech, Hialeah, FL). Harvested viruses were filtered and stored at −80 °C. The virus stocks were routinely tested for contamination with proviral or plasmid DNA by PCR and, if necessary, were treated with RNase-free DNase. Stocks were routinely tested for contamination with proviral or plasmid DNA. Harvested viruses were filtered and stored at −80 °C. Cells were fixed in PBS, 2% paraformaldehyde and analyzed by flow cytometry on a FACScan using CellQuest software.

RESULTS

The Surface of HIV-1 Target Cells Has a Reductive Function That Is Suppressed by PDI Inhibitors—To demonstrate the reductive function of the cell surface, in particular its ability to cleave disulfide bonds in membrane-bound peptides, we measured the release of acid-soluble [32P]tyramine-SH from surface-bound [125I]tyramine-SS-poly(L-lysine) (25). The cationic conjugate was bound to the anionic surface of U937 cells (60 min at 0 °C), and the cells were then incubated for 30 min at 30 °C. The release of radioactivity during incubation was in-
hibited in dose-dependent fashion by three PDI inhibitors that act by different mechanisms. The membrane-impermeant re-
agent DTNB forms mixed disulfides with thiol groups, PAO and its derivative aPAO form coordination bonds through their As3+ with the vicinal thios of the CXXC motif of proteins such as PDI (41), and T3 or AT3 inhibit PDI by binding to sites other than CXXC (42). These agents inhibited the cleavage of both cell-bound conjugate (Fig. 1A) and soluble conjugate exposed to purified PDI (Fig. 1B). In Fig. 1A, the IC50 (in μM) for DTNB, aPAO, and AT3 are 4.9, 5.8, and 70, respectively. In Fig. 1B, the IC50 for DTNB, PAO, and AT3 are 6.8, 12, and 86, respectively. The closeness of these two sets of values measured in two different systems is consistent with the view that in Fig. 1A the conjugate was reduced by surface-bound PDI.

Soluble CD4 Is Coprecipitated by Affinity-labeled Soluble PDI—To determine whether PDI binds to CD4, purified soluble PDI was biotinylated on its thiol with MPDOD. Equimolar amounts of non-biotinylated soluble CD4 were added and mixed, and biotinylated PDI was isolated with immobilized avidin. The two proteins were separated by SDS-PAGE and probed for CD4 with anti-soluble CD4 mAb, and for PDI with mAb RL77. Both proteins were identified on the same blot (Fig. 1C). Control experiments in which sCD4 was isolated with mAb Leu3a and probed with HRP-NeutAviDin revealed no biotiny-
lation, confirming that sCD4 does not contain unconjugated cysteines (43) and indicating that disulfide bonds of sCD4 had not been reduced by PDI.

Cellular PDI Is Coprecipitated by Affinity-labeled Cellular CD4—To test whether a PDI-CD4 complex could be isolated from cell lysate, human 293 cells were engineered to express CD4, CD4-Sep, or SepCD4 coreceptors. CD4-Sep was affinity-purified from the lysate with S-protein-agarose and was found to copurify a significant amount of cellular PDI (Fig. 1D, lane 1). When using cells expressing untagged CD4 but SepCD4-tagged coreceptors, the PDI-CD4 association was maintained and dem-
onstrated through the binding of CD4 to coreceptor SepCD4. After affinity purification of the coreceptor, the eluted proteins were resolved by SDS-PAGE and the Western blots were probed for CD4, stripped, and reprobed for CD4. Again, both proteins were identified (Fig. 1D, lanes 2 and 3). When this experiment was repeated using CD4-negative but CCR5-Sep CD4-expressing cells, PDI was not copurified by the tagged coreceptor (Fig. 1D, lane 4), indicating that PDI does not bind directly to coreceptors.

Surface Proteins Biotinylated on Their Thiols Coprecipitate Small Amounts of Cellular CD4—PDI is a prominent cell sur-
face protein and is the only vicinal thiol-containing surface enzyme to have been positively identified (44). Knowing from Fig. 1D that a PDI-CD4 complex can be isolated from target cell

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lysate, we tested whether thiol-biotinylated surface proteins could isolate CD4. U937 cells were treated with the membrane-impermeant and thiol-specific biotinylating agent MPDOD. Tagged proteins were isolated from cell lysate with immobilized avidin and resolved by SDS-PAGE. The Western blots were probed for PDI, stripped, and reprobed for CD4. Both lysates (D and E), A, reduction of [125I]tyramine-SS-polylysine bound to U937 cells. The decreased release of acid soluble [125I]tyramine-SH caused by increasing concentrations of PDI inhibitors is expressed as percent inhibition. Data are the means ± S.E. from 3 to 6 experiments. (●), DTNB; (■), aPAO; and (○), AT3. B, reduction of the same conjugate in solution by purified PDI and its inhibition by DTNB (●), FAO (■), and AT3 (○). C, coprecipitation of sCD4 with purified biotinylated PDI. The biotinylated enzyme was isolated with immobilized avidin and identified with anti-PDI mAb. Coprecipitated sCD4 was identified on the same blot with CD4 anti-serum. D, lane 1, coprecipitation of untagged cellular PDI by affinity-labeled cellular CD4-Spep (CD4-Spep) gives the signals of total PDI and CD4 in target cells; lanes 2 and 3, bottom, isolation of untagged cellular CD4 coprecipitated by affinity-labeled coreceptors CCR5-Spep (RS-Spep) and CXCR4-Spep (X4-Spep); lanes 2 and 3, top, isolation of untagged PDI coprecipitated by untagged cellular CD4; lane 4, CD4-negative control cells expressing CCR5-Spep showing that PDI is not coprecipitated by CCR5-Spep alone. The Spep proteins were purified from lysates of 293 cells with S-protein-agarose. PDI was probed with mAb RL77, and the stripped blots were reprobed for CD4 with mAb Sim-2, because the cellular form of CD4 has an electrophoretic mobility similar to that of PDI. E, coprecipitation of a small amount of cellular CD4 with biotinylated surface proteins. Thiol sites on the surface of U937 cells were biotinylated with membrane-impermeant MPDOD, and proteins were isolated from the cell lysates with immobilized avidin and resolved by SDS-PAGE. Western blots were probed for PDI, stripped, and reprobed for CD4.

gp120, treated as in lane 5, was biotinylated to label newly generated thiols, probing with HRP-NeutAvidin revealed more than two bands, indicating that disulfide bonds other than V3 were also cleaved (data not shown). Lanes 1, 2, and 4 establish that proteolytic cleavage alone (lane 1), or reduction alone with 2-ME (lane 2) or PDI (lane 4) do not fragment gp120. The top band in lanes 1 and 4–6 corresponds to a gp120 oligomer that is dissociated by 2-ME (lanes 2 and 3). Env and its fragments are detected with polyclonal anti-gp140 Ab.

**Discussion**

**Disulfide Reduction of gp120 and HIV-1 Entry**

To test whether disulfide bonds in recombinant gp120 were accessible to reduction by soluble PDI, we focused on the disulfide bond forming the V3 loop. It is known that thrombin causes a single proteolytic cleavage near the tip of the V3 loop and that this cleavage, if followed by a disulfide reduction at the base of the loop splits gp120 into a 70- and 50-kDa fragment (33). We confirmed this fragmentation using reduction with 2-mercaptoethanol (2ME) (Fig. 2, lane 3). When 2ME was replaced by PDI (1.0 mM GSH), a similar albeit partial fragmentation was obtained (lane 5) showing that PDI cleaves the V3 disulfide bond. GSH alone used as control had no effect. When

**Fig. 1.** Reductive function detected at the surface of HIV-1 target cells (A) and coprecipitation of PDI and CD4 from solution (C) and from cell lysates (D and E). A, reduction of [125I]tyramine-SS-polylysine bound to U937 cells. The decreased release of acid soluble [125I]tyramine-SH caused by increasing concentrations of PDI inhibitors is expressed as percent inhibition. Data are the means ± S.E. from 3 to 6 experiments. (●), DTNB; (■), aPAO; and (○), AT3. B, reduction of the same conjugate in solution by purified PDI and its inhibition by DTNB (●), FAO (■), and AT3 (○). C, coprecipitation of sCD4 with purified biotinylated PDI. The biotinylated enzyme was isolated with immobilized avidin and identified with anti-PDI mAb. Coprecipitated sCD4 was identified on the same blot with CD4 anti-serum. D, lane 1, coprecipitation of untagged cellular PDI by affinity-labeled cellular CD4-Spep (CD4-Spep) gives the signals of total PDI and CD4 in target cells; lanes 2 and 3, bottom, isolation of untagged cellular CD4 coprecipitated by affinity-labeled coreceptors CCR5-Spep (RS-Spep) and CXCR4-Spep (X4-Spep); lanes 2 and 3, top, isolation of untagged PDI coprecipitated by untagged cellular CD4; lane 4, CD4-negative control cells expressing CCR5-Spep showing that PDI is not coprecipitated by CCR5-Spep alone. The Spep proteins were purified from lysates of 293 cells with S-protein-agarose. PDI was probed with mAb RL77, and the stripped blots were reprobed for CD4 with mAb Sim-2, because the cellular form of CD4 has an electrophoretic mobility similar to that of PDI. E, coprecipitation of a small amount of cellular CD4 with biotinylated surface proteins. Thiol sites on the surface of U937 cells were biotinylated with membrane-impermeant MPDOD, and proteins were isolated from the cell lysates with immobilized avidin and resolved by SDS-PAGE. Western blots were probed for PDI, stripped, and reprobed for CD4.

**Fig. 2.** Reduction by soluble PDI of the gp120-disulfide bond forming the V3 loop. Recombinant gp120 used was incubated in the presence of the following reagents. Lane 1, thrombin alone (Th); lane 2, 5% 2ME; lane 3, thrombin followed by 2-ME (Th-2ME); lane 4, PDI alone (50 μg/ml in 1.0 mM GSH); lane 5, thrombin followed by PDI (Th PDI), which followed by GSH alone failed to fragment gp120; lane 6, PDI followed by thrombin (PDI Th). Thrombin followed by 2ME (lane 3) or by PDI (lane 5) cause fragmentations of gp120 in 70- and 50-kDa moieties. When gp120 was treated first with PDI followed by thrombin, more than two fragments were detected (lane 6). Used alone, thrombin (lane 1), 2ME (lane 2), and PDI (lane 4) do not fragment gp120. The top band in lanes 1 and 4–6 corresponds to a gp120 oligomer that is dissociated by 2ME (lanes 2 and 3). Env and its fragments are detected with polyclonal anti-gp140 Ab.

**Disulfide Bonds in gp120 and gp140 Are Reduced upon Binding to the CD4 Receptor of Target Cells**—To test whether disulfide bonds of gp120 are reduced upon binding to cell surface CD4, we used the fact that fresh preparations of gp120 do not have unpaired cysteines (27) and hence no thiols susceptible to biotinylation as verified in control experiments. Therefore, the generation of gp120 thiols is a reliable indicator of disulfide reduction. Target cells were incubated (90 min at 14 °C) with recombinant Env and biotinylated with thiol-specific Biotin-BMCC. Env proteins were isolated and probed. U937 cells were incubated with recombinant oligomeric gp140mIII (34). After biotinylation, the cells were lysed and gp140 was isolated with rabbit anti-gp140 Ab. Proteins were resolved by SDS-PAGE, transferred, and probed for biotinylation with HRP-NeutAvidin (Fig. 3A, top), stripped, and reprobed with mouse anti-gp120 mAb (Fig. 3A, bottom). In the absence of PDI inhibitors, gp140 was distinctly biotinylated (Fig. 3A, top, lane 1). In the

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presence of inhibitors (50 μM PAO or 100 μM AT3), biotinylation was markedly reduced (top, lanes 2 and 3). The inhibition of biotinylation increased with the duration of exposure to inhibitors and inhibitor concentration (data not shown). Inhibitors did not affect binding of gp140 to surface CD4 as shown by the identical intensities of gp140 bands in all lanes (Fig. 3A, bottom). Alternatively, U937 cells were incubated with gp120inh in the presence or absence of PAO. Protein thiols generated in gp120 (or present on other surface proteins) were biotinylated for isolation of biotinylated proteins with immobilized avidin. The proteins were resolved by SDS-PAGE and probed on Western blots for gp120 (Fig. 3B, top) and PDI (bottom), stripped, and reprobed for CD4 (middle). The marked gp120 biotinylation seen in lane 1, top, was significantly reduced by aPAO (lane 2). Probing for CD4 (middle, lanes 1–3) showed coprecipitation of non-biotinylated CD4 by a biotinylated surface protein, most probably PDI (as shown also in Fig. 1E). The slightly increased isolation of CD4 in the absence of aPAO (middle, lane 1 versus lane 2) was confirmed in several experiments and suggests in lane 1 that CD4 was coprecipitated by both biotinylated PDI and by biotinylated gp120, possibly as a gp120-CD4-PDI complex. The faint band of gp120 seen in lane 2 (top) is also consistent with the formation of such a complex, because it may represent non-biotinylated gp120 coprecipitated by the CD4-PDI complex. Probing for PDI using mAb R77 (bottom) showed comparable biotinylation in all lanes. The biotinylation of PDI in lane 2 indicates that covalent bonding through the maleimido group of Biotin-BMCC displaced the coordination bonding of aPAO to PDI thiols. Note that the aPAO and AT3 concentrations that inhibit cleavage of disulfides in gp120 (Fig. 3, A and B) are within the range of concentrations that inhibit the cleavage of [125I]tyramine-SS-conjugate by soluble PDI (Fig. 1B) or by the cell surface (Fig. 1A).

To test whether gp120 from an HIV-1 strain with a different tropism is similarly reduced and whether coreceptor expression is required for Env reduction, CCR5-tropic gp120JR-FL was incubated with either U87.CD4 cells (Fig. 3C, lanes 1 and 2) or U87.CD4.CCR5 cells (lanes 3 and 4). In the absence of inhibitors, thiol biotinylation was comparable in CCR5-tropic and CXCR4-tropic gp120 (Fig. 3C, compare lane 3 with lane 1, top). The generation of thiols was also comparable when gp120JR-FL was bound to cells that did not or did express CXCR5 (Fig. 3C, lane 1 versus lane 3). Inhibitors reduced biotinylation in both cell lines (lanes 2 and 4). Thiols were likewise generated when CXCR4-tropic gp120inh was bound to cells whose CXCR4 coreceptor had been blocked by its natural ligand SDF-1 or by bicyclam (data not shown) (45). Therefore, reduction does not require the binding of gp120 to a functional coreceptor. In summary, recombinant gp120s of different coreceptor specificities as well as oligomeric gp140 undergo distinct disulfide reduction upon binding to CD4+ cells whether or not the cells express coreceptors. In all cases, reduction is distinctly decreased by PDI inhibitors.

**PDI Inhibitors Prevent HIV-1 Entry**—To determine whether cell surface reductase activity is critical for virus entry, we measured the effect of PDI inhibitors on the accumulation of viral DNA in SupT1 cells infected with HIV-1NL4-3. Cells were incubated with PAO or AT3 for 1 h prior to infection with HIV-1NL4-3 and then incubated in the presence of PAO or AT3 for 1 h after infection. PDI inhibitors decreased biotinylation of gp140 from cells incubated in the absence of inhibitors (lanes 2 and 3, top), decrease biotinylation of gp140 from cells incubated in the presence of PAO or AT3 (lane 1–3, bottom). Bottom panel, PDI bands have comparable intensities in all lanes. C, cleavage of disulfide bonds in gp120inh bound to U87.CD4 cells (lanes 1 and 2) or to U87.CD4.CCR5 cells (lanes 3 and 4). Gp120inh thiols were biotinylated, isolated, and probed for gp120 as in B. Cells that do or do not express CXCR5 show comparable gp120 biotinylation (lanes 1 and 3) and comparable inhibition of biotinylation in the presence of aPAO (lanes 2 and 4).
Disulfide Reduction of gp120 and HIV-1 Entry

Entry of X4-tropic and R5-tropic HIV-1 strains into coreceptor-matched target cells is inhibited in dose-dependent fashion by PDI inhibitors

| Target cell (coreceptor) | Tropism of HIV-1a | PDI inhibitor IC50 ± S.D. (µM) |
|--------------------------|-----------------|-------------------------------|
| P4 (CXCR4+)              | X4              | aPAO  29.7 ± 8.8, AT3 24.8 ± 12.2, DTNB 320 ± 120 |
| SupT1 (CXCR4+)           | X4              | AT3 12.0 ± 2.0, R5 7.2 ± 1.4 |
| PM1 (CXCR4+/CCR5+)       | X4              | AT3 4.7 ± 1.5, R5 4.5 ± 0.2 |
| Primary monocyte derived | R5              | AT3 5.7 ± 1.2, R5 3.3 ± 2.1 |
| Macrophages (CCR5+)      | R5              | AT3 590 ± 380 |

* a The CXCR4 and CCR5-tropic strains were HIV-1NL4-3 and HIV-1JR-FL (1 µg of p24/10^6 cells), respectively.
* b Higher concentration of freshly dissolved DTNB required to reach effects comparable with those of aPAO and AT3 may reflect the lower specificity of DTNB and/or the instability of mixed disulfides between DTNB and PDI thiols.

Table I
Entry of HIV-1 into target cells

minus-strand strong-stop DNA, which is the first product of reverse transcription synthesized after viral entry. Target cells were treated with increasing concentrations of inhibitors for 30 min, and HIV-1NL4-3 was added for 1 h. The cells were washed and incubated in complete medium for 5 h, and their total DNA was extracted and amplified by PCR. PDI inhibitors aPAO and AT3 caused a dose-dependent inhibition of strong-stop DNA accumulation in SupT1 cells (Fig. 4). Parallel amplification of a cellular α-tubulin gene was performed to ensure that equal amounts of DNA were in each sample. The autoradiograms of Fig. 4A were quantified, and the decreases in accumulation were plotted as percent inhibition (Fig. 4B). The dose-inhibition relationships are identical for aPAO and AT3. The inhibitors had no effect on the accumulation of DNA when added after infection but were inhibitory when added shortly prior to and/or during infection (Fig. 4C). This indicates that infection requires that the cell surface reductase be active at the time of virus-cell interaction. AT3, aPAO, and DTNB caused dose-dependent inhibitions of HIV-1 entry in a broad range of cells including P4, PM1, H9, 1G5, and macrophage-depleted peripheral blood monocyte cells (Table I) (data not shown). They also inhibited infection by the CCR5-tropic MSLV HIV-1JR-FL in both PM1 and primary monocyte-derived macrophages (Table I). These data complement those of Fig. 3C by showing that the effects of inhibitors on both virus entry and gp120 reduction are independent of gp120 cytoprotosis.

PDI Inhibitors Do Not Impair Entry of an Amphotropic MLV Envelope-pseudo-typed HIV-1—Pseudo-typed virions containing the envelope glycoproteins of amphotropic MLV (aMLV) and the HIV-1 core (35) were used to examine whether or not the inhibitors were specific for HIV-1. SupT1 cells were infected with aMLV envelope-pseudo-typed HIV-1 in the presence of AT3 or aPAO (both 100 µM). Neither inhibitor prevented the infection (Fig. 4D, lane 4, shown for AT3), whereas the entry of native HIV-1 was inhibited (lane 2). Similarly, PDI inhibitors did not prevent infection of 2T3 fibroblasts by ecotropic murine leukemia virus-pseudotyped MSLV expressing green fluorescent protein (data not shown) (36). These results indicate that the reductive activity of the cell surface is not required for entry by two murine leukemia virus envelope proteins but is essential for HIV-1 entry. The normal infection by aMLV-pseudo-typed HIV-1 in Fig. 4D, lane 4, also indicates that PDI inhibitors do not interfere with the reverse transcription of HIV-1.

The following control experiments were performed to confirm the finding that PDI inhibitors prevented HIV-1 entry. 1) Uninfected cells were included as negative controls, and DNA from 8E5/LAV cells (containing a single integrated copy of HIV-1LAV) were used as positive controls in the PCR amplification experiments. 2) The accumulation of amplified reverse transcript was tested in standard 8E5/LAV lysates and was within the linear range of the PCR assay. 3) Inhibition of HIV-1 entry was not caused by virion inactivation, as inhibitor-pre-treated virions retained >90% infectivity after inhibitor removal by ultracentrifugation. 4) Inhibitors did not affect the activity of HIV-1 reverse transcriptase in an in vitro assay. 5) Under the conditions used in the experiments of Fig. 4, DTNB, aPAO, and AT3 were not cytotoxic as measured by trypan blue exclusion and MTT cleavage assays. 6) Neither AT3 nor aPAO inhibited the expression of CD4, CXCR4, or CCR5 on target cells (Fig. 5A). 7) Inhibitors did not decrease HIV-1 binding to target cell CD4 in a whole virion binding assay (Fig. 5B), consistent with the finding that they do not impair binding of gp120 to target cells (Fig. 3A, bottom).

PDI Inhibitors Prevent Infection of P4 Cells by HIV-1NL4-3—To confirm that our data on the inhibition of HIV-1 entry correlate with inhibition of infection, we examined the effect of inhibitors on the ability of HIV-1NL4-3 to transactivate the LTR-LacZ reporter gene present in P4 cells. Both AT3 and aPAO inhibited virus-induced β-galactosidase activity in a dose-dependent fashion (Fig. 6A). This assay requires a 23-h incubation of P4 cells at 37°C after exposure to inhibitors for 30 min prior to and 60 min during infection. As aPAO is not entirely membrane-impermeant, its effects on intracellular PDI (or other proteins containing vicinal thiols) may have caused some additional inhibition. This view is supported by results obtained with GSAO, a membrane-impermeant derivative of aPAO (45) that yielded a dose-inhibition relationship with negligible inhibition at the lowest doses (Fig. 6A, dotted curve). Other controls showed that at the concentrations used, neither inhibitor affected the activity of HIV-1 Tat as Tat-mediated viral transcription was normal in P4 cells pretreated with inhibitors and scrape-loaded with Tat (data not shown).

PDI Inhibitors Prevent Envelope-mediated Cell-Cell Fusion—The effects of PDI inhibitors were also detected at the level of Env-mediated cell-cell fusion in a system that measured transactivation of a LTR-luciferase reporter gene in 1G5 cells upon their fusion with latently infected J1.1 cells expressing HIV-1 Tat (38). The exposure of the fusion partners to inhibitors for 6.5 and 6 h, respectively, caused a significant inhibition of cell-cell fusion (Fig. 6B). This was confirmed in an additional assay measuring the transactivation of the LTR-LacZ reporter gene in P4 cells upon their fusion with HL2/3 cells that express Tat (data not shown) (39). These results are in keeping with the recent report by Fenouillet et al. (46) that the PDI inhibitors used previously to inhibit HIV-1 infection (26) prevent syncytia formation among HIV-1-infected cells. Finally, it is worth noting that the dose-related inhibitions of viral entry, of infection, and of cell-cell fusion (Figs. 4B and 6, A and B) occur at concentrations (50–100 µM) that inhibit disulfide reduction in gp120 bound to CD4 (Fig. 3, A–C) and that inhibit the reductive activities of the cell surface (Fig. 1A) and of soluble PDI (Fig. 1B).
FIG. 5. PDI inhibitors have no effect on CD4 and coreceptor expression or on virus binding to CD4. A, lack of effect of AT3 on the expression of CD4, CXCR4, and CCR5. FACS analysis of PM1 cells labeled with FITC-anti-CD4, FITC-anti-CXCR4 mAb, or FITC-labeled secondary Ab-tagging anti-CCR5 mAb. The expression of the three proteins is identical in the presence (dashed line) or absence (solid line) of inhibitor. B, lack of effect of AT3 on the binding of HIV-1 to SupT1 cells. HLA-DR- SupT1 cells were incubated in the presence or absence of AT3, exposed to HLA-DR+ HIV-1NL4-3 and tagged with fluorescein-labeled anti-HLA-DR mAb. The fluorescence intensity (FL1-H) is measured by FACS and expresses virion binding. Cells not exposed to virus or exposed to virus but treated with mAb Leu3A to block virus binding show comparably low fluorescence intensity (controls, two peaks on left). Cells exposed to virus in the presence or absence of AT3 show comparably high fluorescence (test, two curves on right). The four curves from left to right represent as follows: solid black, no virus; dashed gray, virus added to Leu3A-treated cells; dark gray, virus added to untreated cells; light gray, virus added to cells treated with AT3.

DISCUSSION

The major finding that PDI inhibitors prevent HIV-1 entry strengthens the hypothesis that surface PDI plays a role in HIV-1 infection. The well established reductive function of surface PDI (13–21) together with the putative presence of disulfide bonding close to the domain of gp120-C4D interaction (26) suggested that PDI might cleave disulfide bonds in a gp120 molecule bound to the cell surface. The data of Fig. 3, A–C, show that gp120 indeed undergoes disulfide reduction upon binding to its surface receptor. This reaction is prevented by concentrations of PDI inhibitors that also prevent HIV-1 entry, implying that disulfide reduction in gp120 is required for entry. Although the participation of an unidentified surface reductase has not been formally ruled out, all available evidence points to PDI as the enzyme responsible for reducing gp120.

The selectivity of aPAO and AT3 for PDI is demonstrated by the fact that T3 (the precursor of AT3) was used to isolate PDI from the surface of mammalian cells (47), whereas GSAL (the membrane-impermeant derivative of aPAO) was used to identify surface PDI in mammalian cells lysates (44). DTNB, a nonspecific thiol blocker that has an inhibitory effect comparable to that of aPAO (Fig. 1, A and B) (Table 1), has an inhibitory effect similar to that of anti-PDI antibodies in systems measuring HIV-infection (26) or measuring the fusion of HIV-1-infected cells (46). It indicates that strict specificity of binding to PDI is not required for an inhibitor like DTNB to block the specific oxidoreductive function of PDI. Definitive evidence that PDI is involved in HIV-1 entry will be sought by using engineered target cells that express inactive surface PDI or that overexpress and/or underexpress active PDI. It is known from other studies that changes in expression of intracellular PDI are paralleled at the cell surface (19, 20).

An additional finding is critical to help understand how surface PDI may reach gp120-disulfide bonds, namely how PDI binds to the HIV receptor CD4 (Fig. 1, C and D). The interaction does not prevent the binding of CD4 to gp120 at the cell surface, suggesting that the ectodomain of CD4 has separate binding sites for the two proteins. As gp120 binds to the outermost CD4 domain (D1), it appears probable that PDI binds to the innermost domains (D3 or D4). The proximity of these binding sites enables CD4 to bring PDI to gp120. Because the peptide-binding domains of PDI do not overlap with its active site (42), the latter remains free to interact with gp120. Func-
Table II

Proximity of loop-forming gp120 cysteines to gp120 residues binding to CD4

| Cys-forming loops in gp120 | gp120 contact points with CD4 | Distances to Cys |
|-----------------------------|-----------------------------|------------------|
| V1 (131–157)                | Leu-125                     | 6                |
| V1/V2 (126–196)             | Leu-125                     | 1                |
| V1/V2 stem (119–205)        | Leu-125                     | 6                |
| V3 (296–331)                | Glu-279                     | 17               |
|                             | Glu-380                     | 16               |
|                             | Glu-386                     | 1               |
|                             | Ala-281                     | 15               |
|                             | Thr-283                     | 13               |
|                             | Ser-365                     | 13               |
|                             | Gly-366                     | 12               |
|                             | Gly-367                     | 11               |
|                             | Gly-370                     | 8                |
|                             | Asp-376                     | 10               |
|                             | Glu-370                     | 8                |
|                             | Ile-371                     | 7                |
|                             | Thr-455                     | 10               |
|                             | Arg-456                     | 11               |
|                             | Asp-457                     | 12               |
|                             | Gly-458                     | 13               |
|                             | Gly-459                     | 14               |
| V4 (385–418)                | Asn-425                     | 7                |
|                             | Met-426                     | 8                |
|                             | Trp-427                     | 9                |
|                             | Gln-428                     | 10               |
|                             | Lys-429                     | 11               |
|                             | Val-430                     | 12               |

*Based on data from Fig. 2d of Ref. 48.

As to the accessibility of gp120-disulfide bonds to PDI-mediated reduction, it is demonstrated by the ability of soluble PDI to cleave disulfide bonds in recombinant gp120 (Fig. 2). We had previously suggested that the disulfide bonds forming the V3, V4, and V4/C4 loops were situated in the area of gp120-CD4 interaction (26). The presence of disulfide bonds in this area has been confirmed by structural data published by Kwong et al. (48). These data identify 26 gp120 amino acid residues that make contact with CD4 (see Fig. 2D in Ref. 48). We noticed that 22 of them are situated in the proximity of six gp120-disulfide bonds (Table II), three of which are shown in the gp120 surface representation of the CD4 binding site of gp120 (Fig. 7). The spatial relationship of these three disulfide bonds to gp120-CD4 contact points helps understand how a CD4-bound PDI molecule may reach a gp120-disulfide and may account for the enzymatic gp120 reduction demonstrated by Fig. 3, A–C. It is not yet known which one of these bonds undergoes the initial thiol-disulfide interchange, a reaction that generates two thiols (22) on an open gp120 loop. Here the unusual concentration of disulfide bonds in the domains represented by Table II and Fig. 7 assumes special importance, because these bonds may become substrates for secondary non-enzymatic exchanges propagated by the two thiols initially generated by PDI. Conceivably, disulfide bonds in closely associated proteins may also participate in these secondary exchanges. Such crowding of

![Fig. 7. Presence of disulfide bonds in the face of gp120 that interacts with CD4.](image1)

![Fig. 8. Working model of HIV-1 entry based on PDI-induced conformational changes in gp120 and gp41.](image2)
disulfide bonds in a gp120 domain that was shown to play a central role in Env-cell interactions (8, 48) therefore has the potential of being an epicenter of conformational changes. The crystallographic data identifying that specific domain were obtained with a gp120 core that had interacted with a fragment of sCD4 (48). Therefore, it is intriguing to speculate that the relatively stable initial conformational changes induced by CD4 in that domain may expose disulfide bonds to reduction by CD4-associated PDI and usher a second set of more profound and dynamic PDI-induced structural rearrangements. The cleavage of structure-stabilizing disulfide bonds in proteins is well accepted as a cause of conformational changes. Evidence that such changes occur in gp120 is provided by the observation that PDI-induced reduction of gp120-disulfide bonds exposes new thymolin-sensitive proteolytic sites (Fig. 2, lane 6). The functional significance of disulfide bonds in native gp120 is documented by mutational studies in which the replacement of V3 and V4 cysteine residues rendered the site of gp160 that connects gp120 to gp41 inaccessible to proteolytic cleavage and abolished virion infectivity (49). We propose that post-binding opening of disulfide loops at the time of infection destabilizes the native Env and activates subsequent Env functions that drive envelope-mediated fusion and HIV-1 entry. These two processes start with the interaction of CD4 with HIV-Env that in the case of cell-cell fusion is expressed at the surface of one fusion partner. Both require prior cleavage of gp120-disulfide bonds made possible by the association of PDI with CD4. Our proposal is consistent with the data of Fenoil-lel et al. (46) who show that PDI inhibitors prevent the fusion of HIV-1-infected cells and the formation of syncytia, a hallmark of HIV-1 infection. Their data are supported and extended by our use of two new inhibitors with recognized affinity for PDI and by our testing of Env-mediated fusion in two different assays. Importantly, our data show that PDI inhibitors do not affect the entry of HIV-1 when the virus is pseudo-typed with an aMLV envelope, indicating that an interaction of PDI with the viral envelope is not necessary for the entry of aMLV but is specifically required for HIV-1 entry.

The significance we attribute to the CD4 association with PDI may appear inconsistent with the observation that certain CD4-independent HIV-1 strains are infective (50, 51). However, mutations that render such strains infective may alter the native Env structure and cause conformational changes that mimic those initiated by PDI-CD4. It has also been shown that CCR5 but not CD4 cells when mixed with Env-expressing cells undergo limited fusion in the presence of sCD4 (52). A participation of PDI in this fusion is not excluded, because it is known that PDI is extensively shed from the surface of mamalian cells (20, 23, 24) and HIV target cells (14). Therefore, soluble PDI may be present in the medium of the mixed cell populations used in the fusion assay. We showed that soluble PDI forms complexes with sCD4 (Fig. 1C), which may bind to surface-expressed Env. Such tripart complexes may allow PDI to cleave Env-disulfide bonds, thereby initiating the structural changes that lead to limited Env-mediated fusion of CD4 cells.

How conformational changes in gp120 activate the fusogenic properties of neighboring gp41 remains an open question. A recent publication (53) provides insight into the way gp41 may bind to CD4 in that domain may expose disulfide bonds to reduction by gp120-associated PDI and activate a second set of disulfide bonds. In addition, conformational changes triggered by disulfide bond reduction may uncover novel PDI-induced epitopes of potential use for vaccine development.

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Addendum—A recent publication (55) confirms our finding that an arslenical compound, known to block the vicinal thiols of the CXXC motifs in the active sites of both PDI and thioredoxin, prevents HIV-1 entry. It reports that a disulfide reduction in CD4 (D2) is critical for virus entry. This disulfide reduction is attributed to secreted thioredoxin, a reductase not identified at the cell surface and unable to reduce disulfide bonds in gp120. The article does not link the CD4-disulfide reduction to molecular events that would change the conformation of gp120 or otherwise activate the virus-cell fusion process. Surprisingly, the publication does not discuss the possibility that the inhibition of HIV entry it describes may be attributed to the inhibition of the CXXC-containing surface PDI.

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