



# The Catalytic Activity of Protein Disulfide Isomerase Is Involved in Human Immunodeficiency Virus Envelope–Mediated Membrane Fusion after CD4 Cell Binding

Emmanuel Fenouillet, Rym Barbouche, Joël Courageot, Raymond Miquelis

## ► To cite this version:

Emmanuel Fenouillet, Rym Barbouche, Joël Courageot, Raymond Miquelis. The Catalytic Activity of Protein Disulfide Isomerase Is Involved in Human Immunodeficiency Virus Envelope–Mediated Membrane Fusion after CD4 Cell Binding. *Journal of Infectious Diseases*, 2001, 183 (5), pp.744-752. 10.1086/318823 . hal-03872326

**HAL Id: hal-03872326**

**<https://amu.hal.science/hal-03872326>**

Submitted on 31 May 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# The Catalytic Activity of Protein Disulfide Isomerase Is Involved in Human Immunodeficiency Virus Envelope–Mediated Membrane Fusion after CD4 Cell Binding

Emmanuel Fenouillet,<sup>a</sup> Rym Barbouche,<sup>a,b</sup>  
Joël Courageot, and Raymond Miquelis

Centre National de la Recherche Scientifique, Faculté de Médecine  
Nord, Marseille, France

Protein disulfide isomerase (PDI) is a multifunctional protein with thiol-disulfide redox-isomerase activities. It catalyzes thiol-disulfide interchange reactions on the cell surface that may cause structural modifications of exofacial proteins. PDI inhibitors alter human immunodeficiency virus (HIV) spread, and it has been suggested that PDI may be necessary to trigger HIV entry. This study examined this hypothesis by using cell-to-cell fusion assays, in which the HIV envelope (Env) expressed on the cell surface interacts with CD4<sup>+</sup> lymphocytes. PDI is clustered at the lymphocyte surface in the vicinity of CD4-enriched regions, but both antigens essentially do not colocalize. Anti-PDI antibodies and 2 inhibitors of its catalytic function altered Env-mediated membrane fusion at a post-CD4 cell binding step. The fact that the PDI catalytic activity present on lymphocytes is required for fusion supports the hypothesis that catalysts assist post-CD4 cell binding conformational changes within Env.

The protein disulfide isomerase (PDI) family is composed of proteins that contain  $\geq 1$  domain of  $\sim 100$  amino acid (aa) residues derived from the cytoplasmic protein thioredoxin. A key feature of these proteins is that they exhibit  $\geq 1$  domain with and 1 without the redox-active Cys-X-X-Cys group. This is related to their ability to exert a redox-isomerase function (reviewed in [1]). PDI, a homodimeric protein of 510 aa residues, contains 2 copies of the domains described above. This abundant soluble protein is localized in the endoplasmic reticulum. It catalyzes disulfide bond formation and reduction and thiol-disulfide interchange [1]. At high concentrations, it also behaves as a chaperone that binds incorrectly folded polypeptides to assist protein folding and inhibits protein aggregation. At low concentrations, it facilitates aggregation (antichaperone activity) [2, 3]. For these reasons, PDI is considered to be a multifunctional protein with an important role in the processing of secretory proteins in the endoplasmic reticulum.

Despite having the KDEL endoplasmic reticulum retention signal [4, 5], PDI has been detected in association with the cell surface of various secretory cells (e.g., rat exocrine pancreatic cells [5], platelets [6], lymphoid cells [7], the erythroleukemia

cell line [8], and thyrocytes [9]). Some reports have indicated that cell surface PDI catalyzes thiol-disulfide interchange reactions. This activity may control the functions of extracellular proteins through the induction of structural modifications. For example, PDI influences the cleavage of disulfides required for the activation of the diphtheria toxin through a thiol-disulfide interchange reaction [10], and it mediates functional conformation changes that lead to the shedding of the human thyrotropin receptor ectodomain through the cleavage of disulfide bonds [11].

The mature human immunodeficiency virus (HIV) envelope (Env) is composed of the outer membrane glycoprotein (gp) 120 and transmembrane gp41 subunits. HIV binding to CD4<sup>+</sup> lymphocytes is mediated by gp120 interaction with CD4<sup>+</sup> cells [12]. After CD4<sup>+</sup> cell binding, various gp120 domains interact with various viral coreceptors present on the lymphocyte surface, including chemokine receptors and enzymes [12–14].

Conformational changes within Env follow these interactions between the virus and the surface of the target cells [14]. These post-CD4 binding events are necessary to activate the fusogenicity of gp41 and, consequently, to induce virus–cell membrane fusion and virus entry [15]. These structural modifications are usually thought to result primarily from the intrinsic properties of the Env interacting with HIV receptors. It may be assumed, however, that catalysts also assist in these conformation changes, which may include a partial reorganization of the network of the 10 Env disulfide bonds [16]. Such a hypothetical modification requires a redox-isomerase activity, and PDI is a prime candidate to exert this function. The observation made in a pioneering work by Ryser et al. [17] supports this hypothesis. These authors showed that the catalytic function of PDI is required for HIV spread in cell cultures, since various inhibitors blocking this activity interfere with HIV infection. On the basis of this result,

Received 10 July 2000; revised 21 November 2000; electronically published 25 January 2001.

Financial support: Agence Nationale de Recherche sur le SIDA (2000–2001 to E.F.).

<sup>a</sup> E.F. and R.B. contributed equally to this work.

<sup>b</sup> R.B. is an associate scientist of the Agence Nationale de Recherche sur le SIDA.

Reprints or correspondence: Dr. Emmanuel Fenouillet, Centre National de la Recherche Scientifique, Faculté de Médecine Nord, Bd P. Dramard 13015, Marseille, France (fenouillet.e@jean-roche.univ-mrs.fr).

The Journal of Infectious Diseases 2001;183:744–52

© 2001 by the Infectious Diseases Society of America. All rights reserved.  
0022-1899/2001/18305-0009\$02.00

they postulated, but did not show, that PDI is necessary for virus-cell fusion and HIV entry. Their work [17] is the only one on the role of PDI in HIV infection, and it is not clear why so much time has elapsed without further studies on this topic. Several years after the discovery of the coreceptors, however, the molecular basis of the receptor-induced conformational changes that take place in Env remain to be elucidated. The role that PDI plays in Env conformation changes is intriguing and deserves further study.

## Materials and Methods

**Reagents.** We used PDI (Pierce) and anti-PDI rabbit polyclonal antibodies (SPA-890; StressGen). LY181984 was a generous gift from Lilly Laboratories [18]. Chemical products were of analytical grade (Sigma). VV9-1 is a recombinant vaccinia virus (rVV; gift of M. P. Kieny, Transgène S. A.) that encodes the native fusogenic HIV<sub>Lai</sub> Env [19]. VV1163 is an rVV that encodes a soluble form of gp160<sub>Lai</sub> resulting from the disruption of both cleavage sites present on the native gp160 precursor and from the elimination of the transmembrane domain of the gp41 subunit; consequently, this antigen does not associate on the cell surface [19].

**Confocal laser scanning microscopy (CLSM).** We cultured human lymphoid CEM, MolT4, and C8166 CD4<sup>+</sup> cells in RPMI 1640 medium, supplemented with 5% fetal calf serum (FCS) and 1% glutamine (Gln). In 2 of 4 experiments, cells were activated by the phaseolus vulgaris leucoagglutinin lectin (PHA; Sigma; 5  $\mu$ g/mL) for 12 h [20, 21]. After a wash step, cells were incubated with blocking buffer (PBS; 10% bovine serum albumin [BSA]) and then were incubated with the anti-PDI antibodies SPA890 (1:50 in PBS–0.1% BSA) for 1 h at 4°C [9, 22]. After a wash with PBS–10% BSA, anti-rabbit antibodies (1:100) coupled to Texas red dye (Jackson ImmunoResearch) were added for 1 h. The mouse monoclonal antibody (MAb) CD4 OKT4 (1:30; Ortho Diagnostics Systems) and anti-mouse antibodies (1:100) coupled to fluorescein isothiocyanate dye were used to label CD4 cells. Cells then were washed, were fixed in 4% (wt/vol) paraformaldehyde for 15 min, and were mounted on slides in Mowiol (Calbiochem). Samples were submitted to CLSM (Leica TCS 4D; Leica Lasertechnik).

We monitored a focal series of 8–10 horizontal section planes (0.5  $\mu$ m apart) for each staining. The individual 8-bit-encoded 512  $\times$  512 pixel images, 1 for each channel for each section plane, were visualized with a true color display screen and were processed for analysis of colocalization. A single horizontal section plane representative of the cell sample was used for illustration. To assess fluorescence colocalization, the 512  $\times$  512 pixel images corresponding to the single horizontal section plane used for illustrations were processed with our own computer analysis software. This method selects the regions of true overlap of green and red staining from the non-colocalized staining and allocates white dots to pixels corresponding to the regions where a yellow (red-green colocalization) signal is detected.

**Aggregation and cell-to-cell fusion assays.** CEM cells cultured in 48-well plates (3  $\times$  10<sup>5</sup> cells/300  $\mu$ L) were infected by VV9-1 (2 pfu/cell), as described elsewhere [23]. Various molecules (Bacitracin, 5,5'-dithiobis 2-nitrobenzoic acid [DTNB], PDI, anti-PDI antibodies, and LY181984 at concentrations indicated below) were added

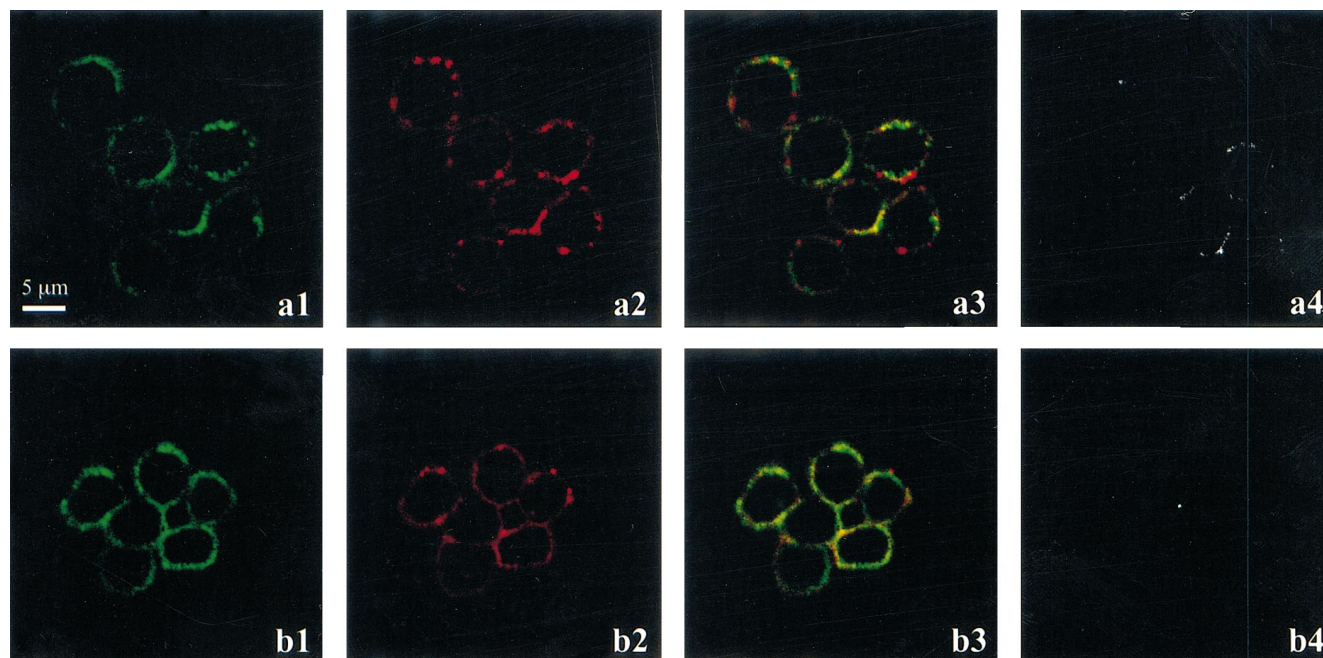
to wells 5 h after rVV infection and before Env-mediated cell aggregation and cell-to-cell fusion appeared in the control well, which was free of inhibitor throughout the experiment. We obtained similar data when the compounds were added together with VV9-1 at the onset of the experiment.

We assessed the ratio (percentage) of the total surface occupied either by cell aggregates (before syncytia appeared) or by syncytia (24 h after infection) versus the surface of the field of vision in 5 fields of vision as follows. First, we scanned a photograph of each field of vision. Then, with Adobe Photoshop software (Adobe Systems) and our own computer analysis software, the areas occupied by aggregates or syncytia were outlined on the photograph, and the sum of the corresponding surfaces was calculated and plotted versus the surface of the field of vision. The cytotoxicity of DTNB and bacitracin was assessed by a thiazolyl blue (MTT) assay (Sigma): cells were incubated with MTT (0.5 mg/mL) for 3 h at 37°C. The reaction was stopped with a lysis buffer (50% dimethylformamide and 20% SDS, pH 4.5). The amount of MTT formazan product was determined (570/655 nm).

In some experiments, CEM or baby hamster kidney (BHK-21) cells (10<sup>5</sup> cells/mL) were infected by VV9-1 (1 and 5 pfu/cell, respectively) in 24-well plates, as described elsewhere [23–25], and were incubated with PDI inhibitors 8 h later. CD4-negative BHK-21 cells were cultured in Glasgow medium supplemented with 5% FCS, 5% tryptose phosphate, and 1% Gln. Such low cell densities do not enable cell aggregation or syncytium formation at post-infection day 2 in CEM cell culture [26]. Cell supernatants were removed 36 h after infection to prevent (see also [24, 25]) the subsequent infection by VV9-1 of the MolT4 fusion partner cells (5  $\times$  10<sup>5</sup> cells/mL) that expressed the various HIV receptors that were added in the presence or absence of inhibitors for another 10–15 h. Cell aggregates and fusions were assessed as described above. Finally, C8166 cells chronically infected with HIV<sub>MN</sub> were washed and were incubated in 96-wells plates (10<sup>4</sup> cells/100  $\mu$ L) [27]. MolT4 cells (5  $\times$  10<sup>4</sup> cells/100  $\mu$ L) were then added in the presence or absence of DTNB. Syncytia were counted 18 h later, as described elsewhere [27].

**Detection of production of Env species by Western blot.** To express Env, CEM cells were infected with VV9-1 (2 pfu/cell) in the presence or absence of DTNB (10<sup>–3</sup> M). Cell pellets (10<sup>6</sup> cells) were washed once in PBS (pH 7.4) and were lysed in sample buffer, as described elsewhere [26]. Cleared lysates and cell-free supernatants were analyzed by 10% SDS-PAGE. Gels were blotted onto a nitrocellulose filter. After blocking with PBS–2% casein (PBSC) and 0.5% Tween 20 for 1 h, the nitrocellulose strips were incubated for 1 h at 25°C with a pool of human HIV-positive or HIV-negative serum samples (1:100) [23, 28]. Incubations and washes were done in PBSC and 0.5% Tween 20. After 2 washes, staining was performed by using peroxidase-coupled antibodies (1:100; Dakopatts) and diaminobenzidine.

**Detection of expression of Env at the cell surface.** CEM cells were infected as described above. After a wash with PBS, cells were incubated for 2 h at 25°C with various dilutions (1:30–1:10,000 in PBSC) of a pool of HIV-positive serum samples to detect membrane Env, as described elsewhere [24, 25, 29]. The use of a pool of serum samples instead of MAbs to quantify surface Env expression avoids potential drawbacks due to immunoreactivity changes within Env in relation to the use of inhibitors [24, 25].



**Figure 1.** Lymphocyte cell lines, CD4, and protein disulfide isomerase (PDI). Living CEM cells (*a*, control conditions; *b*, cells activated by phaseolus vulgaris leucoagglutinin) were incubated with mouse anti-CD4 monoclonal antibody (Ab) or rabbit anti-PDI polyclonal Abs. Staining was done with anti-mouse Abs coupled to fluorescein isothiocyanate (green; column 1) or anti-rabbit Abs coupled to Texas red (red; column 2), respectively. Both labelings are shown in column 3. Processing of images in column 3 enabled selection of regions of true overlap of green and red staining from non-colocalized staining; regions of overlap are shown as white dots (column 4).

Incubations and washes were performed in PBSC in the presence of 0.1% NaN<sub>3</sub>, which blocks endocytosis [26, 29] and enables antibody binding to the cell surface Env to be monitored. To assess whether the signal induced by the pool of HIV-positive serum samples depended on the presence of cell surface-associated Env, we used the following controls: VV9-1-infected cells were incubated with a pool of HIV-negative serum samples and VV1163-infected or VV1163-uninfected cells were incubated with the pool of HIV-positive serum samples. After a wash step, peroxidase-coupled antihuman antibodies were added (1:100 in PBSC) for 1 h. After 2 washes, we added ortho-phenylenediamine to the cells. We measured the absorbance (492 nm/620 nm) of the cell supernatant 30 min after cell pelleting, to prevent interference with absorbance measurement. These conditions enable specific detection of surface-associated Env in an antibody concentration-dependent manner [23, 24, 29].

**Env and CD4 binding assay.** Soluble CD4 (gift of I. M. Jones via Medical Research Council [United Kingdom] Reagents Program) was labeled as described elsewhere [26]: 5 μg was incubated for 30 min at 25°C in a 4-nM iodogen-coated tube with 250 μCi of <sup>125</sup>I-labeled Na. The labeling reaction was stopped by the addition of an excess of tyrosine. The labeled antigen (30 μCi/μg) was purified by chromatography. CD4<sup>+</sup> BHK-21 cells were infected with VV9-1 (10 pfu/cell) for 2 days, to enable a high level of Env expression. Cells (1.5 and 3 × 10<sup>6</sup>) that expressed equivalent amounts of cell surface Env (determined as described above), regardless of the presence of DTNB during Env synthesis, were washed in PBS and were incubated for 2 h at 37°C with <sup>125</sup>I-labeled

CD4 cells (3 × 10<sup>5</sup> cpm/100 μL) in PBSC–0.1% NaN<sub>3</sub>. After 3 washes, cell-associated radioactivity was assessed. Uninfected and VV1163-infected cells were similarly processed, to determine the background binding of CD4 to cell surface exhibiting no Env species. The specificity of <sup>125</sup>I CD4 cell labeling was also assessed by incubating VV9-1-infected cells with soluble CD4 (1 μg/10<sup>6</sup> cells) for 30 min before the addition of <sup>125</sup>I-labeled CD4.

## Results

**PDI is present near CD4-enriched regions on lymphoid cell surfaces.** We verified whether the target of the PDI inhibitors was present on the surface of the CD4<sup>+</sup> lymphocytes used in our assays, and we investigated the surface distribution of CD4 and PDI labeling. About 80% and 50% of CEM cells exhibited a readily detectable CD4 (green) and PDI (red) labeling, respectively (figure 1). The labeling obtained by using the anti-CD4 antibody was rather homogeneous and was present on ~50%–70% of labeled cell surfaces. In contrast, the labeling obtained with the anti-PDI antibodies was organized in small clusters on the cell surface.

To study whether the surface expression of PDI depended on the state of the lymphocyte activation, lymphocytes were treated with PHA in conditions that induce CEM cell activation [20, 21]. This treatment did not substantially modify the intensity of the labeling of PDI, but it increased the CD4 cell labeling

(figure 1). PDI and CD4 were also detected on the surface of C8166 and MolT4 cells (data not shown). Of note, in the cell lines, PDI labeling was usually near the cell surface regions where CD4 cell labeling was important. However, PDI and CD4 exhibited essentially no colocalization on the cell surface, as indicated by the low level of yellow (red-green colocalization) signal on the true color display screen of our equipment in the surface regions where both labelings coexisted. The data shown in figure 1 were processed to further demonstrate the regions of true overlap of anti-PDI and anti-CD4 cell labeling. With this approach, the low level of colocalization of CD4 and PDI is immediately obvious (figure 1).

**PDI inhibitors, cell aggregation, and cell-to-cell fusion.** The catalytic activity of PDI was specifically [17] inhibited by anti-PDI antibodies and by 2 membrane-impermeant PDI inhibitors, DTNB and the antibiotic, bacitracin. Both have a narrow specificity: DTNB is a sulfhydryl blocker that covalently reacts with the catalytic sites of PDI and abolishes its capacity for cleaving disulfides, whereas bacitracin inhibits both its reductive and oxidative functions [11, 17, 30]. We first determined whether these inhibitors could inhibit the Env-mediated cell-to-cell fusion process in human lymphocyte cultures infected by an rVV encoding mature native Env on the cell surface.

This assay is reliable for addressing both Env bioactivity and its ability to interact with a cell surface presenting the various membrane components required for membrane fusion [31]. It enables the efficient discrimination between Env binding to CD4 cells and the membrane fusion step [19, 23, 26]. Indeed, the presence of cell aggregates depends on the interaction of cell surface-associated gp120 with CD4, as shown elsewhere [26]. In brief, the relevance of these aggregates in terms of gp120 and CD4 interaction is shown by the fact that these formations are blocked [26] when Env is expressed by using an rVV coding for a soluble form of Env that does not associate with the cell surface (soluble gp120 and soluble gp160 encoded by VV1132 and VV1163, respectively [19]), and not when soluble CD4 (1  $\mu\text{g}/100\ \mu\text{L}$ ) is added into the cell culture medium or when the anti-CD4 neutralizing Leu3a antibody (500 ng/100  $\mu\text{L}$ ) is present in the cell medium.

When Env was expressed at the cell surface by use of an rVV coding for fusogenic Env (VV9-1) and when fusion partner cells expressed surface CD4 in a context of various HIV coreceptors [31], aggregates between Env-expressing cells and fusion partners developed in a few hours into large multinucleated cells (syncytia). These syncytia specifically reflect the coreceptor-dependent gp41-mediated membrane fusion process, as demonstrated by the following data. First, the presence of anti-V3 neutralizing antibodies interfered with cell-to-cell fusion but not with cell aggregation. Second, large aggregates, but no syncytium, appeared when Env was expressed on the cell surface by a gene coding for a nonfusogenic Env. The latter resulted from disruption of both gp160 cleavage sites (which impaired the activation of the gp41 fusogenicity [VV1139] [19]), removal of

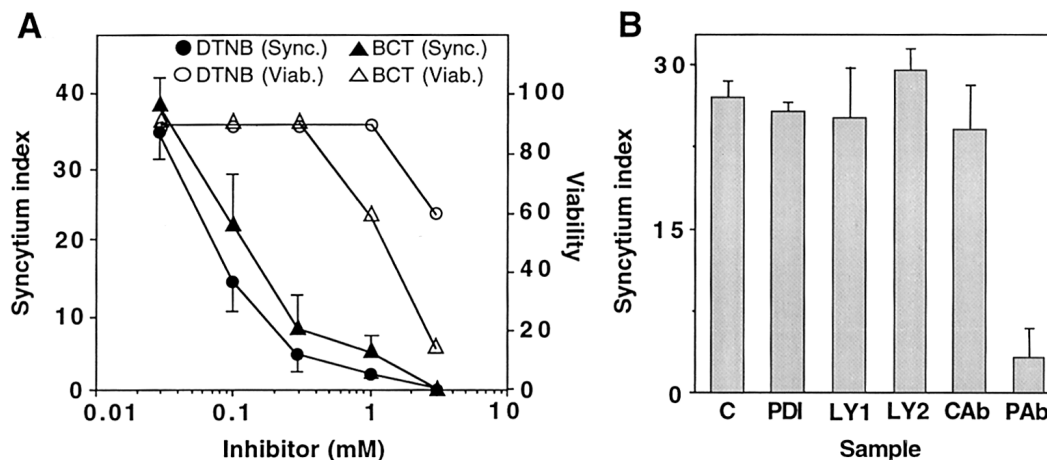
the cleavage site (REKR→NEHQ, which is essential for unmasking the gp41 fusion peptide [VV1134] [19]), and elimination of the gp41 fusion peptide (VV1136) [19]. These control procedures were carried out during development of the assays, to assess routine experimental procedures and to determine the reliability of the test format (see [26] for details).

In each experiment, we also determined the specificity of the assay. First, cell aggregation was inhibited by 70%–80% by the addition of soluble CD4 (1  $\mu\text{g}/\text{well}$ ). Second, infection by VV0 (a VV vector coding for a membrane Env that exhibits CD4 cell binding capacity but not fusogenicity [23]) induced cell aggregation but not cell-to-cell fusion. Third, infection by VV1163, which codes for soluble gp160, did not induce cell aggregation or syncytium formation.

Compared with control conditions (i.e., CEM cell cultures infected by VV9-1 in the absence of inhibitor), the presence of either bacitracin or DTNB in the culture medium from the time that VV9-1 was added to the cell culture exerted a dose-dependent inhibition of cell-to-cell fusion when cultures were observed 24 h after infection. Inhibition was important at  $3 \times 10^{-4}\ M$  (figure 2A). Syncytium formation was abolished at  $10^{-3}\ M$ , in contrast to cell aggregation, which remained unchanged. Similar data were obtained when cells were infected by VV9-1 in the absence of inhibitors to allow VV infection to proceed under routine conditions and when anti-PDI compounds were added to the culture 5 h later. Cell viability in uninfected cell cultures was assessed in parallel experiments after 2 days of treatment with either bacitracin or DTNB and by MTT assay and by monitoring cell counts (figure 2A). These parameters were compared with those obtained in untreated cell cultures. DTNB inhibited syncytium formation at concentrations that lacked cytotoxicity, in contrast to bacitracin. For these reasons, we used  $10^{-3}\ M$  DTNB in the following experiments to block cell-to-cell fusion.

The sulfonyl urea compound LY181984 inhibits thiol-disulfide interchange activities associated at the surface of human cell lines in the micromolar range but not that of PDI [18]. Thus, its lack of effect on syncytium formation at concentrations of  $10^{-4}$  to  $10^{-10}\ M$  (figure 2B shows results at  $10^{-4}$  and  $10^{-6}\ M$ ) argues against a specific role for PDI in fusion. Direct evidence of the involvement of PDI in the fusion process was shown with anti-PDI antibodies (1:50), which inhibited cell-to-cell fusion, in contrast to irrelevant (preimmune) rabbit antibodies; cell aggregation remained unchanged. We also tested the effect of the presence of recombinant soluble PDI in cell medium on cell-to-cell fusion and observed that a high concentration of the molecule (16  $\mu\text{g}/\text{mL}$ ) did not modify syncytium formation.

**Env production and membrane expression in the presence of DTNB.** We examined whether the inhibition by  $10^{-3}\ M$  DTNB of syncytium formation in the system described above was due to the inhibition of rVV infection or to an alteration



**Figure 2.** *A*, Effect on syncytium formation and cytotoxicity of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and bacitracin (BCT). Ratio (percentage) of total surface occupied by syncytia vs. surface of field of vision was assessed (Sync.) in VV9-1-infected CEM cell cultures done in presence of DTNB or bacitracin ( $n = 5$ ; representative experiment shown with means of values obtained in 5 fields of vision; control, absence of inhibitor:  $40\% \pm 5\%$  syncytia). Cell viability (Viab.) was assessed in parallel. *B*, Effect of protein disulfide isomerase (PDI), LY181984, and anti-PDI antibodies on syncytium formation. Syncytium formation was assessed as described above in presence of  $16 \mu\text{g/mL}$  PDI, sulfonamide compound LY181984 (LY1,  $10^{-4} M$ ; LY2,  $10^{-6} M$ ), anti-PDI rabbit polyclonal Abs (PABs), or irrelevant rabbit polyclonal Abs (CABs;  $n = 2$  experiments shown with mean of data from 5 fields of vision; C, control).

of the synthesis, processing, and membrane expression of HIV Env.

**CEM cells express Env with or without DTNB.** Cell pellets were lysed by using sample buffer. Cleared lysates and cell supernatants were analyzed by Western blot. Under either condition, similar quantities of the various Env species were detected in the cell pellet by using a pool of HIV-positive serum samples; gp120 was efficiently secreted into the cell supernatant (figure 3A). The migration profiles of the Env species obtained here were similar to those reported with this expression system [23–25, 29]. The 160-, 120-, and 41-kDa species detected with a pool of HIV-positive serum samples were HIV Env, since a pool of HIV-negative serum samples precluded their detection in samples derived from cells cultured under control conditions.

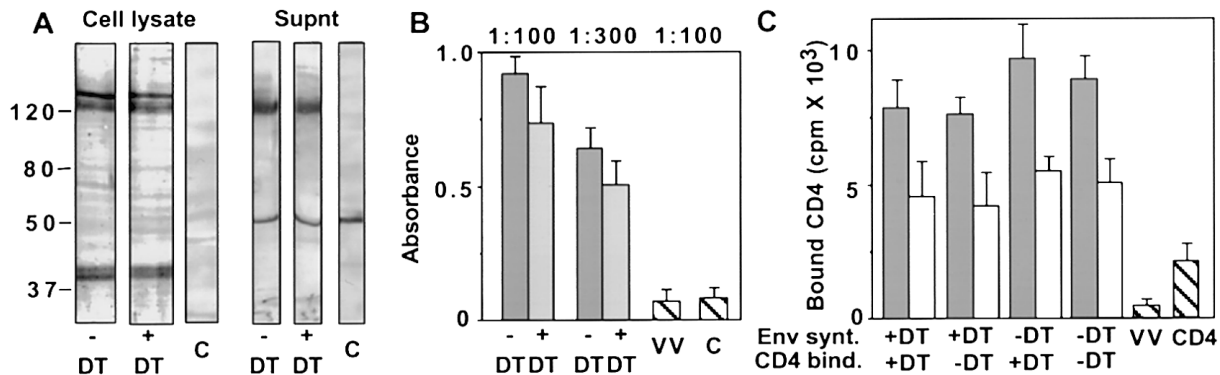
Alternatively, we incubated cells with a pool of HIV-positive serum samples to semiquantify the surface expression of Env [24, 25]. When we used saturating concentrations of serum samples (1:30–1:100), the amount of Env expressed in the presence or absence of DTNB on the surface of the same number of cells led to similar signals (figure 3B). Nonsaturating concentrations (1:300–1:1000) also indicated that the alteration of Env expression by DTNB treatment could not obviously account for the potent anti-Env activity of the compound. Cell surface labeling depends on the presence of surface-associated Env, as demonstrated by the following results. First, the signal obtained by incubation with HIV-positive serum samples of cells infected with VV1163 that codes for soluble gp160 represented 3%–8% of the signal obtained when VV9-1 was used. Second, a similar background signal was obtained when we used either a pool of HIV-negative serum samples to detect Env at the surface of

VV9-1-infected cells (data not shown) or when the HIV-positive serum pool was used for incubation with uninfected cells. These data indicate that  $10^{-3} M$  DTNB lacks important cytotoxicity: the amount of recombinant Env produced in its presence was not significantly affected, and it does not significantly inhibit cell surface expression of mature Env in our conditions.

**DTNB acts on the fusion process at a post-CD4 cell binding step.** We did additional aggregation/syncytium assays, in which DTNB was present during the synthesis of Env and its routing to the cell surface and/or during the coinubation between Env-expressing cells and  $\text{CD4}^+$  fusion partner cells. Our goals were to study whether DTNB exerts its inhibitory activity only during the coinubation between Env-expressing cells and fusion partners and to further determine whether DTNB inhibits gp120 binding to CD4 cells or the membrane fusion step.

Table 1 shows the data when Env was expressed at the CEM cell surface and when fusion partners were MolT4 cells. This format efficiently discriminates between the CD4-gp120 binding step and the membrane fusion step: the specificity of cell aggregation in terms of gp120-CD4 interaction and of syncytium formation in terms of coreceptor- and gp41-mediated fusion were demonstrated as described above and elsewhere [26]. In this assay, the presence of either DTNB (table 1) or anti-PDI antibodies during the coinubation of Env-expressing cells and  $\text{CD4}^+$  fusion partners (contact<sup>+</sup> condition) significantly altered cell-to-cell fusion, compared with control conditions (synthesis<sup>-</sup>/contact<sup>-</sup>). This confirmed the results shown in figure 2A. Moreover, a substantial inhibition of fusion was obtained when MolT4 cells were pretreated for 1 h with either DTNB or anti-PDI antibodies, to block surface PDI before being





**Figure 3.** *A*, Human immunodeficiency virus (HIV) envelope (Env) production in presence of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Env was expressed in presence (+DT) or absence (-DT) of DTNB. Env in cell lysates (Cell) or secreted into cell supernatant (Supnt) was characterized by electrophoresis and Western blot with HIV-positive serum sample pool for staining. In parallel, lysate and supernatant of Env-expressing cells were analyzed with pool of HIV-negative serum samples (C). *B*, Membrane expression. Env was expressed at surface of VV9-1-infected CEM cells with or without DTNB. Semiquantification of membrane Env was done with pool of HIV-positive serum samples (1:100 or 1:300) and then with anti-human antibodies coupled to peroxidase. In parallel, cells infected with recombinant vaccinia virus (VV) encoding soluble gp160 and uninfected cells (C) were similarly processed ( $n = 2$  experiments; duplicates were made). *C*, Labeled CD4 binding (bind.) to Env synthesized in presence (Env synt. +DT) or absence (Env synt. -DT) of DTNB at surface of VV9-1-infected baby hamster kidney (BHK-21) cells (gray column,  $3 \times 10^6$  cells; white column,  $1.5 \times 10^6$  cells) in presence (CD4 bind.: +DT) or absence (CD4 bind.: -DT) of DTNB. Cell-associated radioactivity was counted. Binding to cells ( $3 \times 10^6$ ) infected by recombinant VV encoding soluble gp160 and to cells ( $3 \times 10^6$ ) expressing native Env preincubated with soluble CD4 (CD4) was also determined ( $n = 2$  experiments; duplicates done).

washed and their subsequent incubation with Env-expressing cells for 10 h (Molt4<sup>+</sup> DTNB/wash/fusion); no potent effect was detected when DTNB only was present during Env synthesis (synthesis<sup>+</sup>/contact<sup>-</sup>). These 2 observations are consistent with the idea that the PDI molecules, whose catalytic activity is required for membrane fusion, are present at the surface of the fusion partners and are not those associated with Env<sup>+</sup> cells. The fact that the presence of DTNB only during Env synthesis (synthesis<sup>+</sup>/contact<sup>-</sup>) could not potentially inhibit the subsequent cell-to-cell fusion with fusion partner cells further demonstrates that  $10^{-3}$  M DTNB did not substantially alter the surface expression of mature and bioactive Env (figure 3*A*, 3*B*).

When DTNB was present during coinubation with fusion partner cells, cell aggregation occurred. Similar data were obtained with anti-PDI antibodies. The lack of a potent effect of both DTNB and anti-PDI antibodies on the cell aggregation process is consistent with the results obtained in the VV9-1-infected CEM cell cultures (figure 2). The observation that DTNB did not block the interaction between gp120 and the CD4 cell receptor present on adjacent cells was further illustrated by a molecular CD4-gp120 binding assay: the presence of DTNB during the incubation of Env-expressing BHK-21 cells with <sup>125</sup>I-labeled CD4 cells did not alter CD4 cell binding to membrane Env, compared with control conditions in which DTNB was absent during both Env expression and coinubation with CD4 cells (figure 3*C*). Env expressed in cells treated by DTNB also bound <sup>125</sup>I-labeled CD4 cells.

We controlled the specificity of the CD4 binding reaction several ways. First, the binding of <sup>125</sup>I-labeled CD4 to  $1.5 \times 10^6$  Env-expressing cells was ~50% of that obtained with

$3 \times 10^6$  cells. Second, the binding to uninfected BHK-21 cells or to cells infected with VV1163 represented ~5% of the binding to VV9-1-infected cells. Third, the binding was inhibited by ~70% by preincubation of Env-expressing cells with soluble CD4 (1  $\mu$ g/assay) before the addition of <sup>125</sup>I-labeled CD4. The results obtained with both the aggregation/fusion assays and the molecular CD4 binding assay indicate that DTNB interferes with the cell-to-cell fusion process at a post-CD4 binding step.

This conclusion was further supported by other fusion assays. When Env was expressed at the surface of CD4<sup>-</sup> BHK-21 cells, the fusion between the Env-expressing cells and the fusion partners [26] was inhibited only when DTNB was present during their coinubation. A significant inhibition also was achieved when Molt4 cells were preincubated with DTNB for 1 h before being washed and after incubation with Env-expressing BHK-21 cells. When C8166 cells chronically infected by HIV<sub>MN</sub> were washed and further incubated with Molt4 cells overnight to allow cell-to-cell fusion, a strong reduction (>80%) in syncytium was induced by the presence of DTNB, bacitracin, or anti-PDI antibodies, compared with results in the absence of the inhibitors. The specificity of the syncytium formation was verified by addition of the anti-CD4 neutralizing Leu3a antibody (500 ng/100  $\mu$ L) into the incubation medium, which blocks this process. Inhibition of syncytium formation also was obtained with neutralizing anti-V3 antibodies, as described elsewhere [27]. HIV<sub>MN</sub> infections were also performed as described elsewhere [32]. C8166 cells were incubated for 3 h with serial dilutions ( $10^1$ – $10^{-2}$   $\mu$ L) of a viral supernatant with or without DTNB. Cells then were grown for 12 days. A cytopathic effect correlated with p24 antigen production in the culture medium,

**Table 1.** Effect of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) on cell aggregation and syncytium formation.

DTNB	Synthesis <sup>+</sup> /contact <sup>+</sup>	Synthesis <sup>-</sup> /contact <sup>+</sup>	Synthesis <sup>+</sup> /contact <sup>-</sup>	Synthesis <sup>-</sup> /contact <sup>-</sup>	MolT4 <sup>+</sup> DTNB/wash/fusion
Aggregation index	23% ± 4%	26% ± 4%	24% ± 2%	35% ± 7%	29% ± 7%
Syncytium index	1% ± 1%	3% ± 1%	20% ± 5%	28% ± 5%	5% ± 4%

NOTE. The human immunodeficiency virus envelope (Env) was expressed on surface of CEM cells in the presence (synthesis<sup>+</sup>) or absence (synthesis<sup>-</sup>) of DTNB under conditions that do not enable cell aggregation or syncytium formation. After washing, Env-expressing cells were incubated for 10 h with fusion partner cells in the presence (contact<sup>+</sup>) or absence (contact<sup>-</sup>) of DTNB. Alternatively, fusion partners were incubated for 1 h with DTNB before washing and fusion assay (MolT4<sup>+</sup>DTNB/wash/fusion). Ratio of total surface occupied either by cell aggregates or by syncytia vs. the surface of the field of vision was assessed ( $n = 4$  experiments; representative experiment is shown; means of results obtained in 5 fields of vision are presented).

and p24 antigen production correlated with the virus dose used for infection. HIV infectivity was reduced by ~3 orders of magnitude in the presence of DTNB: p24 antigen production after infection with 10  $\mu$ L of the virus supernatant in the presence of DTNB was lower than that in the absence of DTNB with  $10^{-1}$   $\mu$ L.

The data demonstrate the inhibition by specific PDI inhibitors (anti-PDI antibodies and DTNB) of a post-CD4 binding step of the Env-mediated membrane fusion process in various cell systems in which either recombinant or viral Env interacts with cell surface presenting CD4 in a context of various membrane components required for fusion.

## Discussion

The main objective of this study was to address the postulate that PDI is involved in HIV Env-mediated membrane fusion. Ryser et al. [17] provided convincing evidence that "HIV infection of human lymphoid cells is markedly inhibited by DTNB, by bacitracin, and by anti-PDI antibodies" (p. 4559). The presence of inhibitors of the catalytic activity of PDI during virus-cell interaction in these conditions, however, may have altered HIV infection at several steps of the infectious pathway. Inhibition of HIV entry is likely and can occur at 3 different steps: CD4 binding, the gp41-mediated membrane fusion process, and during uncoating events. It is also possible that HIV expression or budding events may be modified by cell metabolism changes or by cell membrane alterations that result either from entry of the inhibitors into the cells by endocytosis or from cell surface contact with these chemical agents. The first step of HIV infection, CD4 binding, may be impaired by PDI inhibition, inasmuch as this interaction is strongly dependent on the correct folding and disulfide network of both gp120 and CD4. Subsequent to CD4 binding and before virion fusion with the membrane of the host cell, PDI inhibition may also preclude the unfolding of gp120 as part of its interaction with the various HIV receptors (e.g., CXCR4 on CD4<sup>+</sup> lymphocytes). Thus, the gp41-mediated membrane fusion is also a step that may be altered by DTNB, bacitracin, and anti-PDI antibodies, because it requires a stepwise reorganization of the conformation of Env to trigger the ability of gp41 to mediate fusion. Finally, PDI inhibitors may have interfered with virion disassembly,

which releases the reverse-transcription complex. This uncoating step is poorly documented and difficult to study but is highlighted by the use of compounds (catalysts [33], antibodies [34, 35], and bicyclams [36]) that inhibit virus entry without altering CD4 binding or membrane fusion.

We addressed these issues by using biologically sound systems that reasonably mimic the natural interaction of HIV with the native lymphocyte surface. Cell surface-associated native Env was expressed via a vaccinia virus vector [19, 23]. This approach is recommended [31] and is widely used to specifically investigate the fusion mediated by Env and CD4 in the absence of other viral proteins that may skew the results, including those discussed by Ryser et al. [17]. Alternatively, expression of Env at the lymphocyte surface has been obtained by HIV infection.

In our format, membrane Env interacted with adjacent CD4<sup>+</sup> lymphocytes that presented PDI on their surfaces in the vicinity of CD4-enriched membrane regions, as shown here for the first time, and in the context of HIV receptors and fusion partner molecules. First, we investigated whether PDI inhibitors modify the CD4 cell binding capacity of Env. Ryser et al. [17] did not deal with this point but observed that CD4 immunolabeling at the cell surface is unchanged by treating cells with DTNB and that virions pretreated with DTNB can subsequently interact with the CD4<sup>+</sup> cell surface in the absence of DTNB. In contrast, we used semiquantitative assays to show that the presence of PDI inhibitors during Env synthesis or during interaction with CD4 cells had no significant influence on Env binding to the primary viral receptor: aggregation between CD4<sup>+</sup> and Env<sup>+</sup> cells occurred in the presence of either DTNB or anti-PDI antibodies, and DTNB had no effect on Env binding to CD4 at the molecular level. In contrast, when Env was expressed in the absence of DTNB on the cell surface, the presence of the PDI inhibitor or of anti-PDI antibodies during the incubation of Env-expressing cells with CD4<sup>+</sup> fusion partner cells blocked syncytium formation. The various steps of the biosynthetic pathway of Env, including Env synthesis, processing, and membrane expression, apparently were unchanged by the inhibitor. This shows that both DTNB and anti-PDI antibodies act on the HIV receptor-dependent gp41-mediated fusion process per se through inhibition of a post-CD4 binding step.

Our data are also consistent with the hypothesis that the PDI involved in membrane fusion was on the target CD4<sup>+</sup> lym-



phocytes: syncytium formation was inhibited when fusion partner cells were pretreated with either DTNB or PDI antibodies before being washed and before subsequent incubation with Env<sup>+</sup> cells, whereas no significant effect was observed when cells expressing Env were similarly pretreated with DTNB. Moreover, the lack of an effect of soluble PDI on syncytium formation supports the notion that only surface-associated PDI is involved in fusion.

The fact that DTNB exerts its anti-HIV effect by inhibiting the catalytic activity of PDI is supported by the following observations: 2 other inhibitors of the catalytic activity of PDI (PDI antibodies and bacitracin) interfered with syncytium formation in various systems, whereas LY181984, which inhibits some disulfide thiol exchange activities but not that of PDI [18], had no activity in our assays. In addition, the concentration of DTNB that blocked fusion was  $3 \times 10^{-4}/10^{-3}$  M, which is comparable with that active in HIV infection experiments and with that required to inhibit the catalytic activity of PDI [17].

Altogether, our data suggest that lymphocyte surface PDI assists changes in the redox status of HIV Env. This, in turn, may destabilize the gp120 subunit and allow remodeling of the Env disulfide network and induction of profound conformation changes within Env, to trigger membrane fusion, in accord with current models [12, 14, 15].

Finally, we show that PDI labeling is present in the vicinity of regions of the lymphocyte surface enriched in CD4, whereas CD4 labeling also appears in membrane regions where PDI is lacking. This result, together with the finding that PDI catalytic activity is required for the fusion process at a post-CD4 binding step, suggests that only the subset of CD4 receptors present in PDI-enriched surface regions can be involved in HIV into cell entry. If one considers that PDI directly catalyzes disulfide changes within Env after CD4 binding, this suggests that Env binding to CD4<sup>+</sup> cells should subsequently induce the redistribution of PDI and the Env/CD4 complex at the cell surface, to induce their colocalization, and hence contact between the catalyst and Env. Alternatively, it also suggests that Env/CD4 binding and the subsequent membrane remodeling [37] induce the colocalization of PDI and the viral coreceptor CXCR4 at the target lymphocyte surface. Future work will focus on these points. In contrast, if one considers that PDI indirectly influences the redox status of Env through the thiol content of defined membrane regions, such membrane remodeling may not be necessary to trigger fusion.

In conclusion, our results support and expand earlier findings [17] that the catalyst function of PDI at the lymphocyte surface plays a role in HIV entry at the membrane fusion step. We propose that it assists post-CD4 binding Env conformation changes through changes of some of its critical disulfides. Our data also indicate that the cell surface PDI is a novel target for anti-HIV therapies based on new sulfonyl urea compounds with

activity on isomerase functions, such as those already used in anticancer trials [18].

## References

1. Ferrari DM, Soling HD. The protein disulfide isomerase family: unraveling a string of folds. *Biochem J* **1999**; 339:1–10.
2. Gilbert HF. Protein disulfide isomerase. *Methods Enzymol* **1998**; 290:26–50.
3. Gilbert HF. Protein disulfide isomerase and assisted protein folding. *J Biol Chem* **1997**; 272:29399–402.
4. Terada K, Manchikalapudi P, Noiva R, Jauregui HO, Stockert RJ, Schilsky ML. Secretion, surface localization, turnover, and steady-state expression of protein disulfide isomerase in rat hepatocytes. *J Biol Chem* **1995**; 270: 20410–6.
5. Yoshimori T, Semba T, Takemoto H, Akagi S, Yamamoto A, Tashiro Y. Protein disulfide isomerase in rat exocrine pancreatic cells is exported from the endoplasmic reticulum despite possessing the retention signal. *J Biol Chem* **1990**; 265:15984–90.
6. Essex DW, Chen K, Swiatkowska M. Localization of protein disulfide isomerase to the external surface of the platelet plasma membrane. *Blood* **1995**; 86:2168–73.
7. Tager M, Kroning H, Thiel U, Ansorge S. Membrane bound protein disulfide isomerase is involved in regulation of surface expression of thiols and drug sensitivity of B-CLL cells. *Exp Hematol* **1997**; 25:601–7.
8. Zai A, Rudd MA, Scribner AW, Loscalzo J. Cell-surface protein disulfide isomerase catalyzes transnitrosation and regulates intracellular transfer of nitric oxide. *J Clin Invest* **1999**; 103:393–9.
9. Mezghrani H, Courageot J, Bastiani P, Miquelis R. Protein disulfide isomerase in FRTL-5 cells: pH dependent thyroglobulin/PDI interactions determine a novel PDI function in the post endoplasmic reticulum of thyrocytes. *J Biol Chem* **2000**; 275:1920–9.
10. Ryser HJ, Mandel R, Gahni F. Cell surface sulfhydryls are required for the cytotoxicity of diphtheria toxin but not of ricin in Chinese hamster ovary cells. *J Biol Chem* **1991**; 266:18439–42.
11. Couet J, De Bernard S, Loosfeld H, Saunier B, Milgrom E, Misrahi M. Cell surface protein disulfide isomerase is involved in the shedding of human thyrotropin receptor ectodomain. *Biochemistry* **1996**; 35:14800–5.
12. Ugolini S, Mondor I, Sattentau QJ. HIV-1 attachment: another look. *Trends Microbiol* **1999**; 7:144–9.
13. Avril LE, Di Martino-Ferrer M, Pignede G, Seman M, Gauthier F. Identification of the U937 membrane-associated proteinase interacting with the V3 loop of HIV-1 gp120 as cathepsin G. *FEBS Lett* **1994**; 345:81–6.
14. Broder CC, Collman RG. Chemokine receptors and HIV. *J Leukoc Biol* **1997**; 62:20–9.
15. Chan DC, Kim PS. HIV entry and its inhibition. *Cell* **1998**; 93:681–4.
16. Leonard C, Spellman MW, Riddle L, Harris R, Thomas J, Gregory TJ. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *J Biol Chem* **1990**; 265:10373–82.
17. Ryser HJ, Levy EM, Mandel R, DiSciullo GJ. Inhibition of human immunodeficiency virus infection by agents that interfere with thiol-disulfide interchange upon virus-receptor interaction. *Proc Natl Acad Sci USA* **1994**; 91:4559–63.
18. Morré DJ, Jacobs E, Sweeting M, de Cabo R, Morré DM. A protein disulfide-thiol interchange activity of HeLa plasma membranes inhibited by the antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl) urea. *Biochim Biophys Acta* **1997**; 1325:117–25.
19. Kieny MP, Lathe R, Riviere Y, et al. Improved antigenicity of the HIV env protein by cleavage site removal. *Protein Eng* **1988**; 2:219–25.
20. Hasegawa-Sasaki H, Sasaki T. Phytohemagglutinin induces rapid degradation of phosphatidylinositol 4,5-bisphosphate and transient accumulation

- of phosphatidic acid and diacylglycerol in a human T lymphoblastoid cell line, CCRF-CEM. *Biochim Biophys Acta* **1983**;754:305–14.
21. Clevers HC, de Bresser A, Kleinveld H, Gmelig-Meyling FH, Ballieux RE. Wheat germ agglutinin activates human T lymphocytes by stimulation of phosphoinositide hydrolysis. *J Immunol* **1986**;136:3180–3.
  22. Courageot J, Fenouillet E, Bastiani P, Miquelis R. Intracellular degradation of the HIV-1 envelope glycoprotein. *Eur J Biochem* **1999**;260:482–9.
  23. Fenouillet E, Jones IM, Powell B, Schmitt D, Kiény MP, Gluckman JC. Functional role of the glycan cluster of HIV-1 transmembrane glycoprotein gp41. *J Virol* **1993**;67:150–60.
  24. Fenouillet E, Papandréou MJ, Jones IM. Recombinant HIV envelope expressed in an alpha glucosidase I deficient CHO cell line and its parental cell line in the presence of deoxynojirimycin is functional. *Virology* **1997**;231:89–95.
  25. Fenouillet E, Miquelis R, Drillien R. Biological properties of recombinant HIV envelope synthesized in glycosylation mutant CHO cell lines. *Virology* **1996**;218:224–31.
  26. Barbouche R, Decroly E, Kiény MP, Fenouillet E. An anti-human immunodeficiency virus multiple antigen peptide encompassing the cleavage region of the Env precursor interferes with membrane fusion at a post-CD4 binding step. *Virology* **2000**;273:169–77.
  27. Benjouad A, Gluckman JC, Fenouillet E. Effect of sialic acid removal on the antibody response to the third variable domain of human immunodeficiency virus type-1 envelope glycoprotein. *FEBS Lett* **1994**;341:244–50.
  28. Fenouillet E, Gluckman JC. Immunological analysis of human immunodeficiency virus type 1 envelope glycoprotein proteolytic cleavage. *Virology* **1992**;187:825–8.
  29. Barbouche R, Sabatier JM, Fenouillet E. An anti-HIV peptide construct derived from the cleavage region of Env acts on Env fusogenicity through the presence of a functional cleavage sequence. *Virology* **1998**;247:137–43.
  30. Mandel R, Ryser HJ, Ghani F, Wu M, Peak D. Inhibition of a reductive function of the plasma membrane by bacitracin and antibodies against protein disulfide isomerase. *Proc Natl Acad Sci USA* **1993**;90:4112–6.
  31. Ashorn P, Berger EA, Moss B. Vaccinia virus vectors for study of membrane fusion mediated by human immunodeficiency virus envelope glycoprotein and CD4. *Methods Enzymol* **1993**;221:12–8.
  32. Fenouillet E, Gluckman JC, Bahraoui E. Role of N-linked glycans of envelope glycoproteins in infectivity of human immunodeficiency virus type 1. *J Virol* **1990**;64:2841–8.
  33. Fenard D, Lambeau G, Valentin E, Lefebvre JC, Lazdunski M, Doglio A. Secreted phospholipases A(2), a new class of HIV inhibitors that block virus entry into host cells. *J Clin Invest* **1999**;104:611–8.
  34. Corbeau P, Benkirane M, Weil R, et al. Ig CDR3-like region of the CD4 molecule is involved in HIV-induced syncytia formation but not in viral entry. *J Immunol* **1993**;150:290–301.
  35. McInerney TL, McLain L, Armstrong SJ, Dimmock NJ. A human IgG1 (b12) specific for the CD4 binding site of HIV-1 neutralizes by inhibiting the virus fusion entry process, but b12 Fab neutralizes by inhibiting a postfusion event. *Virology* **1997**;233:313–26.
  36. De Clercq E, Yamamoto N, Pauwels R, et al. Potent and selective inhibition of human immunodeficiency virus (HIV)-1 and HIV-2 replication by a class of bicyclams interacting with a viral uncoating event. *Proc Natl Acad Sci USA* **1992**;89:5286–90.
  37. Ugolini S, Moulard M, Mondor I, et al. HIV-1 gp120 induces an association between CD4 and the chemokine receptor CXCR4. *J Immunol* **1997**;159:3000–8.