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Glycosaminoglycans and Protein Disulfide Isomerase-Mediated Reduction of HIV Env

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ABSTRACT

Conformational changes within the human immunodeficiency virus-1 (HIV-1) surface glycoprotein gp120 result from binding to the lymphocyte surface receptors and trigger gp41-mediated virus/cell membrane fusion. The triggering of fusion requires cleavage of two of the nine disulfide bonds of gp120 by a cell-surface protein disulfide-isomerase (PDI). Soluble glycosaminoglycans such as heparin and heparan sulfate bind gp120 via V3 and, possibly, a CD4-induced domain. They exert anti-HIV activity by interfering with the HIV envelope glycoprotein (Env)/cell-surface interaction. Env also binds cell-surface glycosaminoglycans. Here, using surface plasmon resonance, we observed an inverse relationship between heparin binding by gp120 and its thiol content. In vitro, and in conditions in which gp120 could bind CD4, heparin and heparan sulfate reduced PDI-mediated gp120 reduction by approximately 80%. Interaction of Env with the surface of lymphocytes treated using sodium chlorate, an inhibitor of glycosaminoglycan synthesis, led to gp120 reduction. We conclude that besides their capacity to block Env/cell interaction, soluble glycosaminoglycans can effect anti-HIV activity via interference with PDI-mediated gp120 reduction. In contrast, their presence at the cell surface is dispensable for Env reduction during the course of interaction with the lymphocyte surface. This work suggests that the reduction of exofacial proteins in various diseases can be inhibited by compounds targeting the substrates (not by targeting PDI, as is usually done), and that glycosaminoglycans that primarily protect proteins by preserving them from proteolysis also have a role in preventing reduction.

Surface glycoprotein gp120 and transmembrane gp41 subunits together constitute the mature human immunodeficiency virus (HIV) envelope (Env) protein (Einfeld, 1996). HIV binds the CD_4+ lymphocyte surface through gp120 interaction with various ligands such as CD_4 and glycosaminoglycans (GAGs) (Doms and Peiper, 1997; Ugolini et al., 1999). Upon specific binding to CD4, various events, including interaction with the coreceptor CXCR4 (Berger et al., 1999) and reduction of two of the nine disulfide bonds of gp120 (Gallina et al., 2002; Barbouche et al., 2003; Markovic et al., 2004), lead to structural changes within Env that eventually unmask the gp41 fusion peptide, enabling its insertion into the target cell surface to trigger HIV/cell-membrane fusion (Berger et al., 1999; Pierson et al., 2004).

Cell-surface protein disulfide-isomerase activity (PDI) plays a prominent role in the process of HIV entry because its inhibitors block membrane fusion and infection (Ryser et al., 1994; Fenouillet et al., 2001). In the endoplasmic reticulum, PDI catalyzes reduction and oxidation reactions and has an important role in the folding of proteins (Ferrari and Soling, 1999). PDI is also expressed on the surface of the cell, where it can cause redox modifications, hence activation, of exofacial proteins (Mandel et al., 1993). On the lymphocyte surface and after HIV binding, it is responsible for gp120 reduction, which occurs in the context of a complex composed by gp120, PDI, and HIV receptors (Gallina et al., 2002; Barbouche et al., 2003; Markovic et al., 2004).

The positively charged V3 domain of lymphotropic Env binds both cell-surface–associated and soluble polyanions such as heparin and heparan sulfate (Batinic and Robey, 1992; Ugolini et al., 1999). It has been reported that an additional binding site is induced after interaction with CD4

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ABBREVIATIONS: HIV, human immunodeficiency virus; PDI, protein disulfide isomerase; BCT, bacitracin; Env, human immunodeficiency virus envelope glycoprotein; GAG, glycosaminoglycan; GSH, glutathione; HP, heparin; HS, heparan sulfate; MPB, 3-(*N*-maleimidylpropionyl)biocytin; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RU, resonance unit; SH, thiol group; SPR, surface plasmon resonance; Tg, thyroglobulin; CEM, human CD4+ lymphoid cells.

and probably corresponds to the CXCR4 binding domain on gp120 (Moulard et al., 2000). The role of cell-surface–associated GAGs in HIV entry, however, remains unclear. Indeed, there is a report that excludes a major role for GAGs in mediating HIV-1 attachment via interactions with virus gp120 (Zhang et al., 2002). Whereas heparin and related compounds are reported not to interfere with gp120-CD4 binding (Harrop and Rider, 1998; Moulard et al., 2000; Zhang et al., 2002), they neutralize HIV probably at least through interference with CXCR4 interaction (Roderiquez et al., 1995; Rider, 1997; Harrop and Rider, 1998; Moulard et al., 2000). From these observations, soluble sulfated polyanions were used early in the 1990s in clinical trials and are still considered to be potential therapeutic anti-HIV agents (Rider, 1997).

Here, using a procedure that allows controlled chemical reduction of Env and surface plasmon resonance (SPR), we showed that gp120 binding to GAGs depends on its redox state. Using an in vitro assay in which gp120 is specifically reduced upon the addition of PDI, we observed that both heparan sulfate and heparin reduced gp120 disulfide cleavage and that such inhibition was increased when gp120 was preincubated with CD4. Our data indicate that the inhibition of PDI-mediated gp120 reduction may constitute part of the anti-HIV effect of soluble GAGs. In contrast, cells treated with sodium chlorate, which inhibits GAG synthesis, were capable of exerting normal PDI-mediated Env reduction, indicating that cell-surface associated GAGs are dispensable for Env reduction.

Materials and Methods

Reagents. The thiol reagent 3-(*N*-maleimidylpropionyl)biocytin (MPB) was purchased from Molecular Probes (Eugene, OR). Reagents including bovine liver PDI, porcine thyroglobulin (Tg), streptavidin-coupled peroxidase, and bacitracin were purchased from Sigma-Aldrich (St. Louis, MO). Sheep polyclonal antibody D7324 (Aalto, Dublin, Ireland) is an anti-peptide antibody directed against the C terminus of gp120. Heparan sulfate and heparin were from Celsus (Cincinnati, OH) and Sigma-Aldrich, respectively. C-clade gp120 derived from the HIV_{CN54} isolate was expressed using recombinant baculoviruses and purified from the supernatant of infected cells by lectin affinity chromatography. Soluble recombinant CD4 consisting of the D1 and D2 domains of the native antigen was supplied by the EVA-MRC ADP Programme.

Controlled Env Reduction by β-Mercaptoethanol. Env was treated with increasing concentrations of β-mercaptoethanol (0 to 1% for 15 min at 25°C) (Barbouche et al., 2003) in phosphate-buffered saline, pH 7.4 (PBS) before dot-blotting, MPB-labeling, and further processing to assess its thiol content, as described below. For binding experiments, the sample was treated with β-mercaptoethanol and then with iodoacetamide (3:1 iodoacetamide/reductant ratio; 10 min at 25°C) to prevent further reoxidation of the thiol groups, as described by Barbouche et al. (2003). After lyophilization to remove β-mercaptoethanol, the sample was processed for binding to GAGs as described below.

Env Reduction by Soluble PDI. gp120 (2 μg/20 μl of PBS, final volume of the assay) was incubated for 45 min at 25°C in the presence or absence of soluble CD4 (1 μg), PDI (0.02 to 1 μg) and GSH (1 mM final concentration), and GAGs (3 μg of heparin or 6 μg of heparan sulfate; approximately 3 M excess). Otherwise, Env was preincubated for 45 min with CD4 and/or GAGs before the addition of PDI. Bacitracin (1 mM), a PDI inhibitor, was used to assess the PDI-dependence of the reaction. Samples were then dot-blotted and processed as described below and elsewhere (Barbouche et al., 2003).

Env Reduction by Cell-Surface–Associated PDI. Human CD4+ lymphoid cells (CEM; 10^6 cells/ml) were treated, or mock-treated, using 30 mM sodium chlorate for 24 h before trypsin treatment and a further 24-h incubation in the presence of the inhibitor. Cells (3×10^7) were then either treated using 1 mM bacitracin or mock-treated for 1 h. They were then incubated for 1.5 h at 37°C with Env (5 μg/200 μl). Cells were treated by NaN_3 (0.1%) to inhibit further surface remodeling. MPB was added to the cell pellet (0.3 mM for 30 min at 25°C). Excess reagent was blocked using glutathione (0.5 mM for 10 min at 25°C), and remaining sulfhydryl groups in the system were quenched with iodoacetamide (1 mM for 10 min at 25°C) (Barbouche et al., 2003). Cells were washed and incubated in acid buffer (2-morpholinoethanesulfonic acid/HCl 10 mM and NaCl 150 mM, pH 3) for 10 min to dissociate surface-bound Env, as described previously (Barbouche et al., 2003). The eluate was adjusted at pH 7 using NaOH and Env was immunoprecipitated for 4 h at 25°C using D7324 antibody covalently coupled to CNBr-Sepharose CL4B (Pfizer, Inc., Täby, Sweden) as described by Barbouche et al. (2003). After elution using 3% SDS, the purified envelope samples were dot-blotted and processed as described below to determine the corresponding thiol content. In parallel, dot-blot quantification of the amount of gp120 present in the eluate after immunoprecipitation using D7324 was achieved as described in Barbouche et al. (2003). Together, these assays allow MPB reactivity to be related to the amount of immunopurified Env to determine the thiol content per molecule of gp120.

Thiol Content Determination. Samples (a 250-ng aliquot part in 10 μl of PBS) were dot-blotted onto a nitrocellulose filter (Schleicher & Schuell, Paris, France). After blocking with PBS, 2% casein, and 0.5% Tween 20, filters were washed using PBS, 0.5% casein, and 0.5% Tween 20, incubated with MPB (a thiol reagent coupled to biotin whose reaction with the thiols of proteins can be detected using streptavidin peroxidase; 0.1 mM for 30 min at 25°C), and reacted with streptavidin-coupled peroxidase (1:500 for 30 min at 25°C). Labeling was performed using diaminobenzidine, and spot intensity was quantified by densitometry (PhosphorImager; Amersham Biosciences Inc., Piscataway, NJ). Similar results were obtained when incubation with MPB was performed in the vial before dot-blotting (Barbouche et al., 2003). Changes in the thiol content of Env were quantified using a standard curve obtained after dot-blot of increasing amounts of Tg and subsequent MPB labeling as described above and elsewhere (Barbouche et al., 2003). Taking into account Tg thiol content determined as described in Barbouche et al. (2003), we assessed the thiol content of Env present in each protein sample. This assay detects thiols with a sensitivity of 0.3 pmol.

SDS-PAGE Analysis of MPB-Labeled Sample. Env incubated with PDI, CD4, and GSH as described above was treated using 2 mM MPB for 30 min at 25°C. Quenching was performed using GSH (4 mM for 10 min at 25°C) and iodoacetamide (8 mM for 10 min at 25°C). The sample was then analyzed by SDS-PAGE (10%) and, after blotting, processed using streptavidin peroxidase as described above. Purified Env and the batch of PDI used here were similarly analyzed.

Analysis of the Interaction of Env or PDI with GAGs by SPR. Experiments were performed using a Biacore 3000 instrument. Heparin was biotinylated as described in Sadir et al. (2001) and captured on a C1-streptavidin–coupled sensorchip. For this purpose, two flow cells of a C1 sensorchip were activated with 50 μl of a mixture of 0.2 M *N*-ethyl-*N*′-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride/0.05 M *N*-hydroxysuccinimide before injection of 50 μl of streptavidin (0.2 mg/ml in 10 mM acetate buffer, pH 4.2). Remaining activated groups were blocked with 50 μl of ethanolamine 1 M, pH 8.5. In general, this procedure permitted the coupling of approximately 400 resonance units (RU) of streptavidin. Biotinylated heparin (5 μg/ml) in HEPES-buffered saline containing 0.3 M NaCl was then injected over the surface through one of the two flow cells to obtain an immobilization level of 30 to 40 RU. The other flow cell was left untreated and served as a negative control. Flow cells were then conditioned with several injections of 1 M NaCl. For binding assays,

Env in HEPES-buffered saline was simultaneously injected at 15 μl/min onto the streptavidin and the heparin surface for 16 min, after which the complexes formed were washed with buffer. The sensorchip surface was regenerated for 1 min with a pulse of 0.05% SDS and for 5 min with a pulse of 2 M NaCl. Control sensorgrams (measured on the streptavidin surface) were subtracted on line from the sensorgrams obtained with the streptavidin-immobilized heparin to yield true binding responses.

Results

Binding of gp120 to GAGs Is Redox State-Dependent. We determined the relationship between the thiol content of Env and its capacity to bind GAGs. The susceptibility of gp120 to β-mercaptoethanol was first examined. Env was treated with various concentrations of β-mercaptoethanol, and the corresponding free thiol content was determined for each condition (Fig. 1A). We observed that Env from the Lai (laboratory-adapted) isolate and Env from the CN54 (clinical) isolate were similarly sensitive to the reducing agent, although the Lai species was slightly more susceptible to high concentrations of β-mercaptoethanol than its CN54 counterpart. From these data, a precise relationship between the concentration of reductant and the thiol content of Env induced by the presence of the reagent was established, and we prepared samples exhibiting a defined amount of reduced bonds per Env_{CN54}: after treatment with the β-mercaptoethanol concentrations determined previously, aliquots (250 ng) from each condition were processed to confirm the expected thiol content of each sample (Fig. 1B), as described above. The majority of the sample was incubated with iodoacetamide, the reducing agent was removed by lyophilisation, and the resulting Env was assayed for GAGs binding using SPR. Injection of Env (30 nM) over a sensorchip functionalized with 30 RU of heparin gave a specific binding response

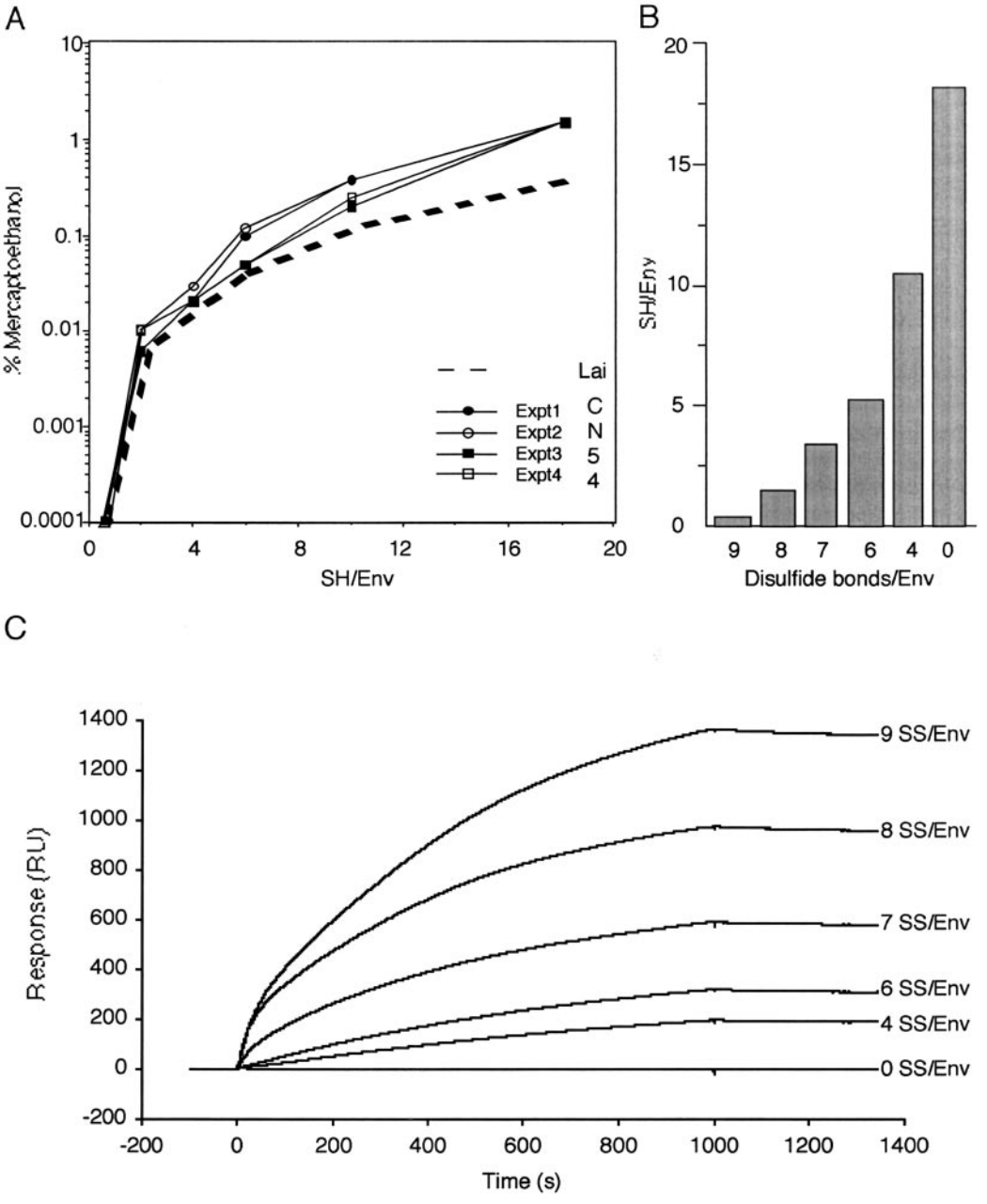

Fig. 1. Redox state of Env and GAG binding capacity. A, chemical reduction of CN54- and Lai-derived Envs. Env treated by increasing amounts of β-mercaptoethanol was blotted onto a nitrocellulose filter and stained using MPB. Labeling was performed using streptavidin-coupled peroxidase and diaminobenzidine. Spot intensity was quantified by densitometry. Using a standard curve obtained as described under *Materials and Methods*, the thiol content per molecule was determined in each condition (CN54, four independent experiments are shown; Lai, two independent experiments with identical results). B, thiol content of the various Env_{CN54} species used in SPR analysis. Based on the data presented in A, Env was treated by increasing amounts of β-mercaptoethanol to obtain populations exhibiting in average ~9, 8, 7, 6, 4, or 0 disulfide bonds per gp120. The thiol content of the samples was characterized as in A. C, overlay of sensorgrams showing binding to immobilized heparin of Env_{CN54} exhibiting various redox states. Envs (30 nM) prepared as described in B were simultaneously injected for 16 min (from 0 to 1000 s) over a streptavidin or a heparin-activated surface at a flow rate of 15 μl/min. The sensorgrams show the specific binding response.

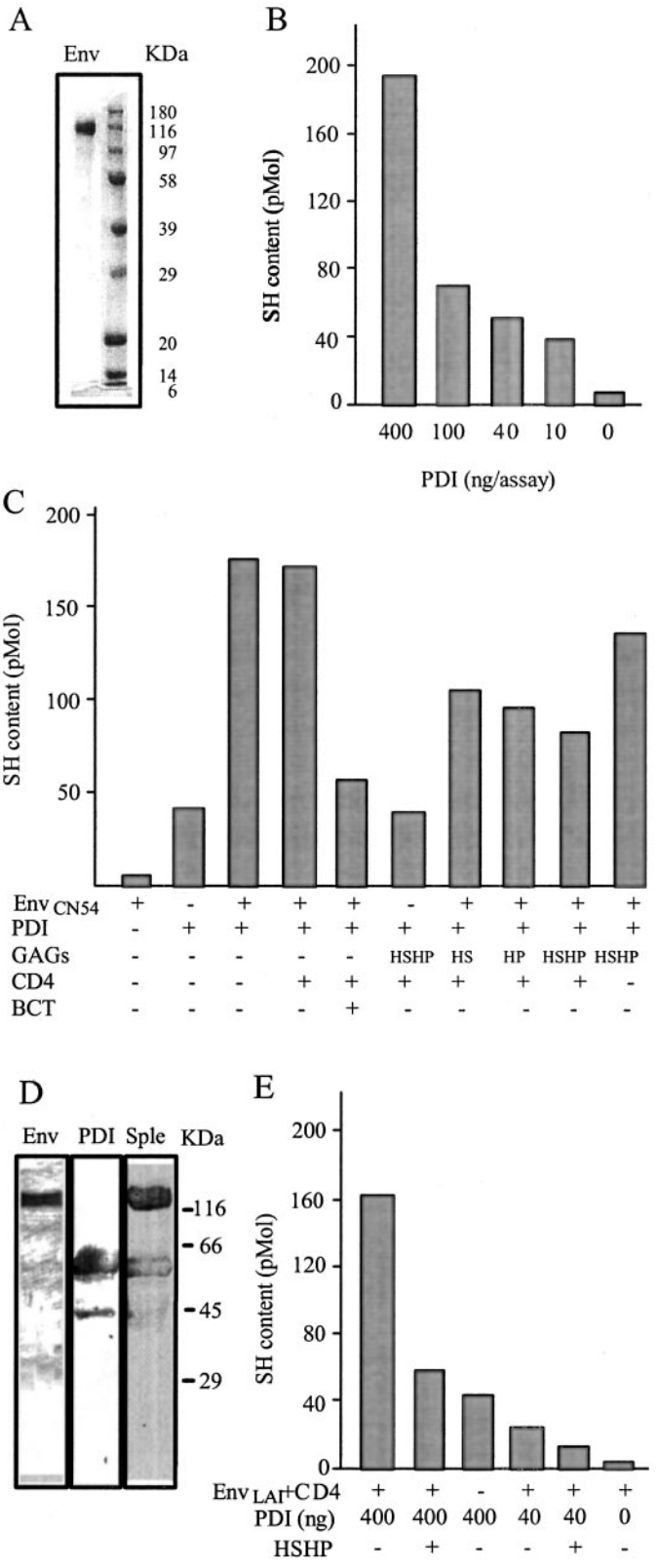

Fig. 2. In vitro Env reduction and effect of GAGs. A, SDS-PAGE analysis of Env_{CN54}. Env was submitted to SDS-PAGE analysis. The gel was Coomassie Blue-stained. B, PDI-mediated Env_{CN54} reduction. Env (2 μg/20 μl, final volume) was incubated with various concentrations of PDI, as indicated. The thiol content of the sample was assessed as indicated under *Materials and Methods* (n = 2; an experiment is shown). C, assessment of the assay and effect of GAGs on Env_{CN54} reduction by PDI. Env was incubated in the presence or absence of CD4, PDI (400 ng/20 μl), or GAGs (HP, heparin; HS, heparan sulfate) as indicated under *Materials and Methods*. Bacitracin (BCT) was used to assess the PDI-dependence of the reaction. Samples were then dot-blotted and stained with MPB and streptavidin peroxidase. Labeling was performed using diaminobenzidine, and spot intensity was quantified by densitometry. The thiol content of the samples was determined using a standard curve obtained as described under *Materials and Methods* [each condition was performed 2 (+BCT) to 7 (+Env+HSHP) times; means are shown]. D, SDS-PAGE analysis of Env_{CN54}, PDI, and the Env+/PDI+/GAG−/CD4+/BCT− sample (Sple). After incubation with MPB, each sample was submitted to SDS-PAGE analysis. Western blotting and staining were achieved using streptavidin peroxidase and diaminobenzidine. E, effect of GAGs on Env_{Lai} reduction by PDI. Env and CD4 were incubated with the reagents (PDI, HSHP) as indicated. Samples were processed as described in C (each condition was performed two times; results from one experiment are shown).

of 1300 RU with unreduced Env and an inverse relationship between Env thiol content and its capacity to bind GAGs for all subsequent samples, with the fully reduced molecule being wholly unable to interact with heparin (Fig. 1C).

Inhibition by GAGs of Env Reduction by PDI. We studied whether GAGs influenced Env reduction by PDI through the establishment of an in vitro PDI-mediated Env reduction assay. Purified (Fig. 2A) C-clade $gp120_{CN54}$, which exhibited binding to CD4 and several conformational monoclonal antibodies suggesting native conformation (I. M. Jones, unpublished data), was assessed for free thiol content in the absence of PDI. It was found to be very low (Fig. 2, B and C), in agreement with a previous report on Env_{Lai} (Barbouche et al., 2003), confirming that essentially all cysteine residues are involved in disulfide bonds on biologically active gp120. Preliminary experiments measuring the extent of free thiols after incubation with PDI showed that the maximum, albeit still partial (see below), gp120 reduction was obtained using 20 μg/ml (Fig. 2B). At this concentration of PDI, a significant increase in the thiol content of the sample was obtained compared with the control (Fig. 2, B and C). Pre- (data not shown) or coincubation of Env with CD4 did not modify the signal induced by PDI compared with that resulting from Env/PDI incubation alone. The occurrence of free thiols was PDI-specific because it was substantially inhibited in the presence of 1 mM bacitracin, an antibiotic that blocks both the reductive and oxidative capacity of PDI (Mandel et al., 1993). Omission of the Env component from the assay led to only weak signals, probably resulting from the PDI active thiols groups, because SH groups are absent on heparin and heparan sulfate, and a sample containing only 400 ng of PDI led to a similar signal. This interpretation was confirmed using MPB labeling and SDS-PAGE analysis of the reactants (Fig. 2D). A sample from the Env+/PDI+/GAGs−/CD4+/BCT− condition shown in Fig. 2C was incubated with MPB and resolved by SDS-PAGE and filter transfer as described previously (Barbouche et al., 2003). The labeling associated with each band was determined by scanning densitometry of the resulting nitrocellulose filter (Molecular Analyst; Bio-Rad, Hercules, CA). We found that most (74%) of the labeling was associated with the 120-kDa Env species, 20% with a band corresponding to the molecular weight of PDI, and 4% with a faster migrating band species that also appeared in the PDI sample. These results confirm the conclusion reached from the comparison of the Env−/PDI+/GAGs+/CD4+/BCT− and Env+/PDI+/GAGs−/CD4+/BCT− conditions presented in Fig. 2C; i.e., 70 to 75% of all MPB-labeled species present after incubation of gp120 with CD4 and PDI

were Env, indicating that it is a specific target for PDI reduction under the conditions used, and CD4 was not reduced. This result enabled us to assess the signal specifically due to the thiol content of Env in each of the following conditions: by subtracting from the thiol content of the sample 40 to 45 pmol SH caused by the PDI-associated thiol content and 5 pmol SH caused by the thiol content of the native Env population, we obtained the data presented in Table 1. A 3 M excess of HP and HS in the incubation medium significantly decreased the signal obtained when Env was pre- (data not shown) or coincubated with CD4, with PDI being the last species that was added in the medium (Fig. 2C and Table 1). A weaker inhibition was observed in the absence of CD4. Incubation at 25 or 37°C led to similar results, and incubation of GAGs with Env exhibiting either two or six disulfides after chemical reduction (as described in Fig. 1B) did not prevent its subsequent labeling using MPB (data not shown), indicating that GAGs themselves did not interfere with binding of the reagent to Env in our conditions. From these data (Table 1), we concluded that PDI enabled the reduction of three to four disulfides within Env in vitro, that the presence of CD4 only marginally influenced disulfide cleavage, but that GAGs inhibited the PDI-mediated Env reduction process by approximately 80% in the presence of CD4. Additional controls were performed (data not shown): preincubation of PDI with GAGs and its further 1/50 dilution—to obtain a final concentration of GAGs insufficient for inhibition of Env reduction—did not modify Env reduction, indicating that GAGs did not exert their effect in our assay through direct PDI inhibition; this result is in agreement with our observation that using SPR, no interaction between PDI and heparin and heparan sulfate was observable.

Because R4-Envs may encode a highly positively charged V3 loop (e.g., Lai) and because V3 loops of R5-Envs show low charges (e.g., CN54), the origin of gp120 may influence the susceptibility to inhibition induced by the polyanions. We therefore examined the effect of GAGs on Env_{Lai} reduction and observed an inhibition which was similar to that shown above for Env_{CN54} (Fig. 2E).

A lower concentration of PDI (2 μg/ml) only reduced one to two Env disulfides (CN54, Fig. 2B and Table 1; Lai, Fig. 2E). In these conditions, the resulting thiol content of Env was similar to that observed during the HIV entry process (Barbouche et al., 2003). The signal resulting from the presence of PDI thiol groups was very weak (approximately 2%), and MPB detected essentially only Env-associated thiols. Heparin and heparan sulfate exhibited a similar capacity to inhibit Env reduction under these conditions, with the mixture displaying the strongest inhibition ability (CN54, Table 1). Increased inhibition of Env reduction by GAGs in the presence of CD4 was again noted (CN54, Table 1; Lai, Fig. 2E). Together, these results indicate that the GAGs interaction with Env results in inhibition of gp120 reduction by PDI, and that this inhibition increases after contact with CD4.

TABLE 1

Inhibition by GAGs of the PDI-mediated Env reduction process

According to the data presented in Fig. 2 and in the text, the amount of free thiols induced by PDI on Env was calculated as described in the text. The values reported are expressed as free thiols induced per Env; means are presented; $n = 2$ to 7.

Condition	+PDI 20 μg/ml	+PDI 2 μg/ml
−HP/HS −CD4	7.3	3.0
−HP/HS +CD4	7.0	3.2
+HS −CD4	ND	2.6
+HS +CD4	3.2	1.9
+HP −CD4	ND	1.7
+HP +CD4	2.4	1.3
+HP/HS −CD4	4.9	2.4
+HP/HS +CD4	1.7	1.0

Role of GAGs in the Reduction of Env Disulfide Bonds during the Course of Interaction with the Lymphocyte Surface. We then examined whether GAGs modulate PDI-mediated Env_{LAI} reduction at the cell surface. Preliminary experiments showed that syncytium formation induced by the contact between HIV Env-expressing cells and fusion partner CEM cells was only marginally inhibited after treatment of partner cells with noncytotoxic doses of GAG-degrading enzymes chondroitinase ABC and heparinase I (data not shown). We concluded that such enzyme treatment did not substantially affect the various events required for fusion, including gp120 reduction, which is a prerequisite of fusion (Barbouche et al., 2003), although a role for GAGs that were inaccessible to exogenously added enzymes could not be excluded. Sodium chlorate competes with sulfate for sulfotransferase enzymes and therefore blocks the sulfation of new GAG chains, preventing GAG synthesis. Preliminary experiments, in agreement with a previous report on HIV infection (Patel et al., 1993) and as recommended in the literature, showed that 30 mM sodium chlorate was the optimum nontoxic concentration inhibiting fusion between HIV Env-expressing cells and chlorate sodium-treated CEM cells. Therefore, we investigated the role of lymphocyte surface-associated GAGs in the PDI-mediated gp120 reduction process after treatment with this concentration of inhibitor. After incubation with Env, thiol labeling of the cell-surface–associated components was performed using MPB, the labeled Env recovered from the lymphocyte surface by an acid wash and captured using antibody D7324 coupled to Sepharose, resulting in the recovery of pure cell-surface–modified gp120 (Barbouche et al., 2003). The thiol content and amount of immunopurified gp120 was assessed to determine the thiol/gp120 ratio. Although the quantity of gp120 bound to cells treated for 48 h with sodium chlorate was approximately two thirds of that isolated from the nontreated cells, the extent of Env reduction in both conditions was similar (Fig. 3). The PDI dependence of the observed reduction was shown using bacitracin: the inhibitor prevented the increase of the thiol content observed in its absence, whereas it interfered with neither Env binding to CD4 (Fenouillet et al., 2001) and CXCR4 (Barbouche et al., 2003) nor the reaction of MPB with protein thiols (Barbouche et al., 2003). Thus, cell-surface GAGs seem not to be necessary for Env reduction by the lymphocyte surface.

Discussion

Nine of 10 Env disulfides are present on gp120 (Leonard et al., 1990), and the importance of changes in the redox state of approximately two of these bonds in prefusion events has been outlined recently (Gallina et al., 2002; Barbouche et al., 2003; Markovic et al., 2004). These reports show that gp120 reduction occurs as part of a complex composed at least by CD4, Env, and PDI clustered on the lymphocyte surface. PDI-mediated reduction probably contributes to the confor-

mational changes that eventually trigger Env fusogenicity, because PDI inhibitors block HIV/cell fusion (Ryser et al., 1994; Fenouillet et al., 2001). These observations may be of significant experimental therapeutic relevance (Fenouillet et al., 2004).

During its interaction with the cell surface, gp120 also interacts with GAGs (Mondor et al., 1998; Ugolini et al., 1999). Both the V3 loop (Roderiquez et al., 1995; Rider, 1997) and a conformational region, probably the CXCR4 binding domain (Moulard et al., 2000), have been identified as the binding determinants on lymphotropic gp120s. Env-GAGs binding is believed to proceed from an initial high-affinity association with V3 to a second, CD4-induced, site (Harrop et al., 1994; Roderiquez et al., 1995; Moulard et al., 2000). The initial HIV/GAGs interaction may act to enable HIV to survey the cell surface for CD4/PDI complexes. That GAGs play a major role in mediating viral gp120 attachment to target cell, however, has been reported to be unlikely (Zhang et al., 2002). These data and reports describing the potent anti-HIV effect of heparins and dextran sulfate (Ito et al., 1987; Rider, 1997) indicate that despite their weak concentration on the CD4+ lymphocyte surface, Env interaction with GAGs may have direct biological relevance for HIV entry (Patel et al., 1993; Ohshiro et al., 1996).

For these reasons, we investigated whether soluble and cell-surface–associated GAGs influenced PDI-mediated Env reduction. Using SPR, we examined the relationship between the redox state of Env and its capacity to bind heparin and found that this interaction depends on the level of correct disulfide bonding. These data are consistent with the observation that Env binding to GAGs involves V3 (Roderiquez et al., 1995) but that other disulfide-bridged, conformation-dependent regions may be also involved (Harrop et al., 1994; Moulard et al., 2000). In contrast, CXCR4 binding does not tolerate alteration in the redox state of Env, whereas CD4 binding is abrogated when more than one bond was cleaved (Barbouche et al., 2003).

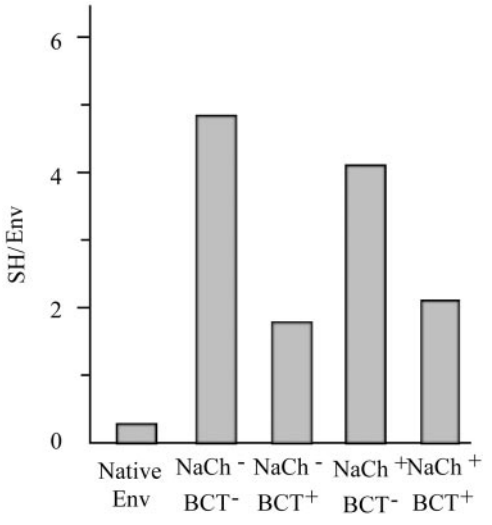

Fig. 3. Reduction of Env_{CN54} disulfide bonds after lymphocyte binding. CEM cells were treated using sodium chlorate (NaCh+; 48 h), bacitracin (BCT+; 1 h) or mock-treated (NaCh−, BCT−). They were then incubated with Env before MPB labeling. Env was acid-dissociated and immunoprecipitated before thiol content assessment (n = 3 measurements; means are shown). The thiol content of Env from the batch we used (Native gp120) was determined in parallel.

We then examined the influence of soluble GAGs on Env reduction. An in vivo study on the effects of adding soluble GAGs to cell-surface-associated PDI-mediated gp120 reduction cannot be carried out because polyanions interfere with initial Env/cell surface interactions (Harrop et al., 1994; Moulard et al., 2000), whereas reduction on the cell surface occurs late during interaction, at a prefusion step (Gallina et al., 2002; Barbouche et al., 2003; Markovic et al., 2004). Thus, we developed an in vitro assay. We used soluble forms of the proteins because the extent of gp120 reduction as part of the surface CD4/PDI/Env complex is similar for both monomeric and membrane-associated oligomeric Env (Barbouche et al., 2003) and GAGs bind both forms of Env (Moulard et al., 2000). CXCR4 was not considered a necessary addition because it does not seem to directly participate in Env reduction, with CXCR4− and CXCR4+ cells reducing Env similarly (Gallina et al., 2002; Markovic et al., 2004). We found that the thiol content of gp120 after incubation with a high concentration of PDI displayed approximately 6 to 8 thiols per molecule compared with <1 thiol in the original population. This result confirms the capacity of PDI to reduce gp120 in vitro (Gallina et al., 2002) and extends this finding by quantifying the extent of the reduction. The finding that an average of 2 Env disulfides are cleaved during HIV binding to lymphocytes is consistent with the present data, because it is likely that here, most soluble Env interacts with PDI, in contrast to what occurs during HIV interaction with the lymphocyte surface. Our data also suggest that Env reduction during HIV interaction with lymphocytes can occur after low-affinity interaction of Env with the cell surface and contact with PDI, irrespective of Env binding to the viral receptors and, therefore, probably as part of an abortive pathway in terms of fusion, because the revelation of thiols on Env precludes CXCR4 binding (Barbouche et al., 2003). It is possible that the increased concentration of PDI in CD4-enriched regions (Fenouillet et al., 2001; Gallina et al., 2002, Markovic et al., 2004) may be used to advantage by the virus to carry out Env conformational changes to achieve fusion competence, with gp120 reduction not necessarily requiring the particular receptor-induced conformation to progress despite this being the norm. In addition, reduction is detected after receptor binding, perhaps as a consequence of the increased probability of contact between Env and PDI within the CD4/Env/PDI complex as part of the process leading to fusion. This would explain the discrepant observations that gp120 reduction was observed after CD4 binding in CXCR4− cells yet after CXCR4 interaction using native lymphocytes (Gallina et al., 2002; Barbouche et al., 2003; Markovic et al., 2004): the tighter the Env/cell-surface interaction, the stronger the reduction.

Examination of the data obtained when Env was incubated with heparin and heparan sulfate in solution showed that PDI-mediated changes in the redox status of gp120 were inhibited, an effect that strongly increased when gp120 bound to CD4. This result is consistent with the induction of an additional GAGs binding domain on Env, besides V3, after CD4 binding (Harrop et al., 1994; Moulard et al., 2000). R4-Envs with highly positively charged V3 loop (e.g., Lai) or R5-Envs presenting V3 with low charges (e.g., CN54) were similarly protected from reduction by the negatively charged GAGs. A similar extent of inhibition of reduction was observed when heparin or

heparan sulfate was added to a concentration of PDI that gave a thiol content of treated gp120 similar to that of the reduced Env population after interaction with the lymphocyte surface (Barbouche et al., 2003). The capacity of polyanions to protect proteins and regulatory polypeptides from enzymatic proteolysis through interaction with the substrate is well known (Saksela et al., 1988; Calatroni et al., 1992; Gupta-Bansal et al., 1995; Sadir et al., 2004). Our study indicates that GAGs can also prevent disulfide cleavage mediated by reductases through interaction with the substrate. Thus, GAGs, besides their capacity to protect proteins from degradation exerted by proteases, can also exert protection through inhibition of their reduction.

Because our work shows that PDI activity can assist the cleavage of disulfide bonds within Env irrespective of its interaction with receptors and that soluble GAGs inhibit this event, we speculated that the disruption of the Env disulfide network by PDI can be controlled on the lymphocyte surface by Env interaction with cell-surface–associated GAGs as part of the multimolecular complex formed by Env, receptors, and catalysts (Fenouillet et al., 2001; Gallina et al., 2002; Barbouche et al., 2003; Markovic et al., 2004). This process may occur during the early stage of a general recruitment of cell-surface and cytoskeletal proteins into a "fusion synapse" that completes entry of the virus into the cell (Jolly et al., 2004). On the other hand, Env interaction with GAGs may be important in protecting disulfide bonds that should not be cleaved during reaction with PDI to protect its fusogenic capacity. The inhibition of GAGs synthesis did not significantly modify the capacity of surface PDI to reduce gp120, indicating that cell-surface GAGs do not play a direct role in gp120 disulfide cleavage.

Our study shows that soluble GAGs can inhibit HIV infection by altering Env reduction by PDI, a prerequisite for fusion, in addition to the direct effect on the interaction of Env with cell-surface components such as coreceptors. Thus, and as illustrated by Fig. 4, if the virus can escape the antiviral effect resulting from coreceptor binding inhibition (Roderiquez et al., 1995; Rider, 1997; Harrop and Rider, 1998; Moulard et al., 2000), its entry process may be blocked as shown here at the latter step of the process triggering membrane fusion (i.e., the reduction process of two of its disulfide bonds). Interference with Env reduction may be exerted either by bridging, hence stabilizing, distant disulfide bonded domains of Env or by steric hindrance after Env binding, so preventing PDI access to the susceptible bonds in Env. Our work also shows that inhibition of disulfide bond reduction by surface reductases can be achieved by compounds that interact with the substrate and not solely with the catalyst, as achieved previously (Mandel et al., 1993). This offers new perspectives in the therapeutic regulation of a process required for activation of various exofacial proteins involved in physiological (Lawrence et al., 1996; Sahaf et al., 2003) and pathological events (Ryser et al., 1991; Droge, 2002).

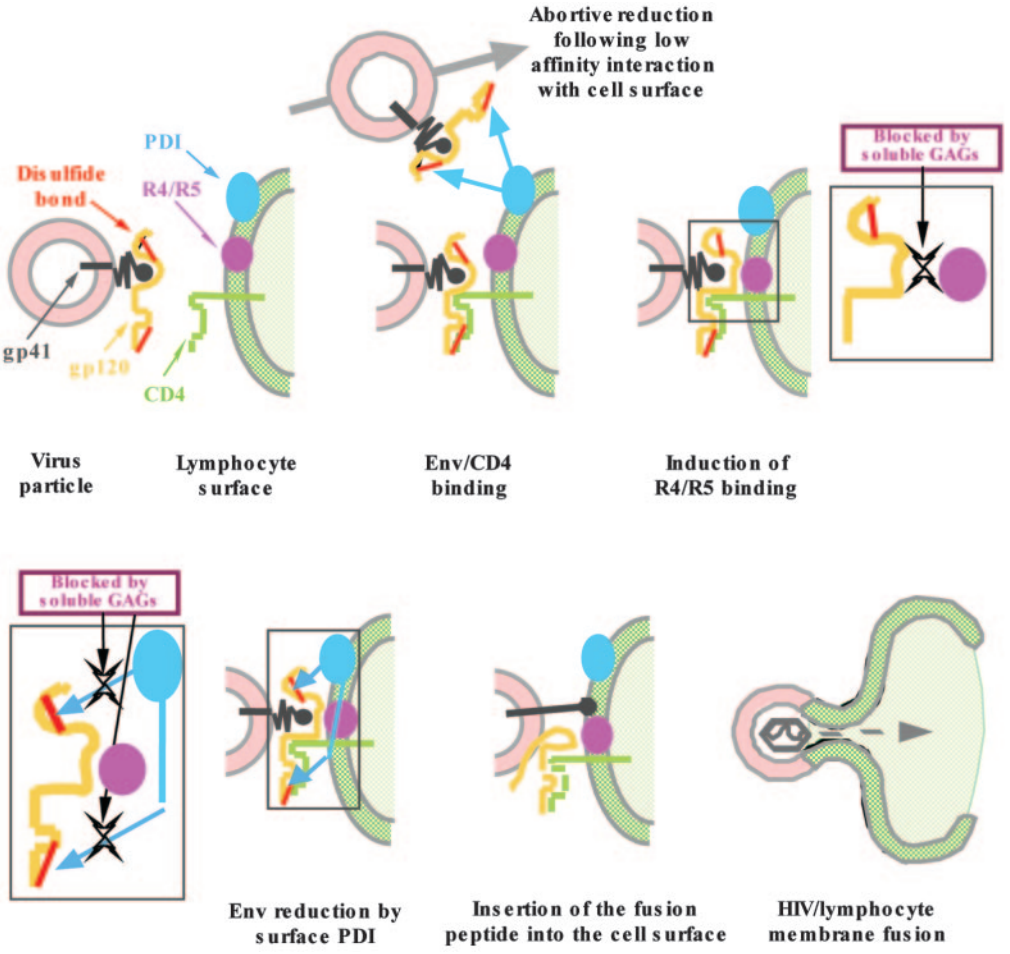

Fig. 4. HIV/lymphocyte interaction and effect of GAGs. The main steps of the HIV/lymphocyte interaction process are shown, as well as the step at which disulfide bond cleavage is believed to occur to enable productive infection. From the data presented here and in articles referenced herein, the potential targets of GAGs are shown.

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