Secreted phospholipases A₂, a new class of HIV inhibitors that block virus entry into host cells

David Fenard, Gérard Lambeau, Emmanuel Valentin, Jean-Claude Lefebvre, Michel Lazdunski, and Alain Doglio

Introduction

HIV-1 infection is initiated by the interaction of the virion envelope complex (gp120/gp41) with at least 2 cellular receptors: the CD4 molecule (1, 2) and a member of the chemokine receptor family (3–6). Subsequent to binding with these cellular receptors, the gp120/gp41 complex undergoes conformational changes that mediate fusion of the viral membrane with the target-cell membrane (7–9). After virus-cell fusion, virion disassembly occurs (uncoating) to release the reverse transcription (RT) complex that dissociates from the plasma membrane and moves toward the cell nucleus (10). This complex contains all the viral functions necessary for the synthesis of the proviral DNA, its transport to the cell nucleus, and its integration into the host cell DNA (11–14).

Mammalian and venom secreted phospholipases A₂ (sPLA₂s) have been associated with a variety of biological effects. Here we show that several sPLA₂s protect human primary blood leukocytes from the replication of various macrophage and T cell–tropic HIV-1 strains. Inhibition by sPLA₂s results neither from a virucidal effect nor from a cytotoxic effect on host cells, but it involves a more specific mechanism. sPLA₂s have no effect on virus binding to cells nor on syncytia formation, but they prevent the intracellular release of the viral capsid protein, suggesting that sPLA₂s block viral entry into cells before virion uncoating and independently of the coreceptor usage. Various inhibitors and catalytic products of sPLA₂ have no effect on HIV-1 infection, suggesting that sPLA₂ catalytic activity is not involved in the antiviral effect. Instead, the antiviral activity appears to involve a specific interaction of sPLA₂ to host cells. Indeed, of 11 sPLA₂s from venom and mammalian tissues assayed, 4 venom sPLA₂s were found to be very potent HIV-1 inhibitors (ID₅₀ < 1 nM) and also to bind specifically to host cells with high affinities (K_a < 1 nM). Although mammalian pancreatic group IB and inflammatory-type group IIA sPLA₂s were inactive against HIV-1 replication, our results could be of physiological interest, as novel sPLA₂s are being characterized in humans.

Mammalian and venom secreted phospholipases A₂ (sPLA₂s; 14 kDa) are found in mammalian tissues and animal venoms and catalyze the hydrolysis of glycerophospholipids to release FFAs and lysophospholipids (22–27). They have been classified into different groups on the basis of the number and position of the cysteine residues present in their sequences (24, 27). So far, 6 mammalian sPLA₂s referred to as group IB, IIA, IIC, IID, V, and X have been cloned and associated with different physiological and pathological processes (25–29). Aside from their function as enzyme, sPLA₂s have been shown to associate with specific membrane

receptors that participate to their biological activities (27). To date, 2 main types of sPLA2 receptors have been identified. N-type receptors are expressed at high levels in brain, but they are also present in other tissues (30–32). These receptors bind with high affinities different venom sPLA2, such as bee venom sPLA2 (bvPLA2) (31). The 180-kDa M-type receptor is expressed in various tissues including lung, kidney, and liver and belongs to the C-type lectin superfamily (27). The M-type receptor has been proposed to be involved in a variety of biological effects of sPLA2 including cell migration, eicosanoid release, and septic shock (33, 34), and recent data have indicated that this receptor is the physiological target for the mammalian endogenous group IB and group IIA sPLA2 (35).

Mammalian sPLA2 are likely to play important roles in host defense (25, 27, 36); sPLA2 products have been shown to interfere with viral infection (37, 38); and venom sPLA2 display a wide and intriguing array of biological effects. We therefore analyzed whether sPLA2 have antiviral properties against HIV-1. Our results indicate that several, but not all, assayed sPLA2 can protect efficiently various host cells from the replication of HIV-1 isolates and are likely to define a new class of HIV-1 inhibitors.

Methods

Plasmids. Plasmids encoding HIV-1 virus (pNLAD8, pYU2, pBru-2), HIV-2 virus (pROD10), and pCMVTat were kindly provided by P. Charneau, K. Peden, and N. Israel, respectively (Pasteur Institute, Paris, France). The human CCR5 chemokine receptor construct (pCCR5) was obtained after insertion of the cloned receptor cDNA in the mammalian expression vector pCEP4 (Invitrogen BV, Groningen, the Netherlands).

Antibodies. Polyclonal antibodies to bvPLA2 (rbvAb) were obtained after immunization of rabbits by 4 successive injections of 350 μg of bvPLA2. These antibodies specifically recognize bvPLA2 in Western blot (working dilution 1:1000) and prevent bvPLA2 antiviral effect (working dilution 1:40). Anti-human CD4 mAb Leu3A was from Becton Dickinson (Pont de Claix, France).

Reagents. Purified HIV-1 gp120 and SDF-1α were from NeoSystem Laboratories (Strasbourg, France) and PeproTech (London, United Kingdom), respectively. OS1, bvPLA2, taipoxin, basic sPLA2 from Naja mossambica mossambica snake venom (Nmm_CMIII), porcine group IB (pGIB), and recombinant human group IIA sPLA2 (hGIIA) were prepared as described (31, 35). Nigexine from Naja nigricollis (39), PA2 and PA5 sPLA2s from the venom of the lizard Heloderma suspectum (40), human group IB sPLA2 (hGIB), and catalytically inactive BaIV sPLA2-like protein from Bothrops asper (41) were kindly provided by André Ménez (C.E.A. Saclay, Gif/Yvette, France), Jean Christophe (Université libre de Bruxelles, Belgium), the late Hubertus Verheij (University of Utrecht, the Netherlands), and José Maria Gutiérrez and Sergio Lizano (University of Costa Rica, San José, Costa Rica), respectively. Oleoyloxyethylphosphocholine was obtained from Calbiochem-Novabiochem (Meudon, France). Other reagents were from Sigma-Aldrich (St. Quentin Fallavier, France).

Cell culture. Activated PBMCs (10⁶ cells/mL) were obtained after treatment of cells with 3 μg/mL phytonagglutinin for 48 hours in RPMI-1640 medium (GIBCO BRL, Cergy Pontoise, France) supplemented with 20% FBS (BioWhittaker Europe, Verviers, Belgium) followed by a 24-hour incubation with 20 U/mL of recombinant human IL-2 (Roche, Meylan, France). MT4s and CEMs are CD4+ T-cell lines obtained from...
sPLA₂s block HIV-1 viral cycle before RT. (a) Time-of-addition experiments with bvPLA₂ and AZT on the replication of HIV-1BRU. P4 cells were incubated with virus at 4°C for 90 minutes to allow virus binding. Unbound viruses were then removed by several washes and cells were shifted to 37°C to allow virus entry and infection. bvPLA₂ (100 nM) or AZT (50 µM) were added at different times after virus addition until 8 hours, after which the cell culture medium was replaced by fresh medium containing AZT. Two days later, the level of viral replication was measured by β-gal assay. (b) sPLA₂ effect on proviral DNA synthesis. MT4 cells (10⁷ cells) were infected with high doses of infectious HIV-1BRU supernatant in the presence of AZT or 100 nM bvPLA₂, NmmCMIII, taipoxin, or pGIB. Early after infection (8 hours), HIV-1 proviral DNA was extracted and analyzed by Southern blot. Each lane was loaded with 10 µg of soluble DNA, and the blot was hybridized with a specific [³²P]-labeled HIV-1 riboprobe. The arrow indicates the position of the unintegrated linear proviral HIV-1 DNA (9.2 kb).

**Figure 2**

**HIV-1 viral stocks and infection assays.** The primary HIV-1 isolates CMU02, 91US054, 91US056, and 92US657 were obtained from the NIH AIDS Research and Reference Reagent Program. These primary HIV-1 isolates were propagated on activated PBMCs and harvested from the medium at the peak times of Gag p24 production. HIV virus stocks were produced by transient transfection of pBru-2, pROD10, pNLAD8, or pYU2 plasmids in the human embryonic kidney 293 cells (CRL-1573; American Type Culture Collection, Manassas, Virginia, USA) using a calcium phosphate mammalian transfection kit (Stratagene, Montigny le Bretonneux, France). Three days after transfection, supernatants were collected and centrifuged (1,500 g for 15 minutes). High-titer viral stocks were obtained by mixing 10⁷ HIV-1BRU-infected MT4 cells with 4 × 10⁵ MT4 cells in 40 mL of culture medium containing 20 µg/mL polybrene (Sigma-Aldrich). The next day, cells were diluted to 2.5 × 10⁶ cells/mL and further incubated for 1–2 days until cell lysis was about 50%. Infectious supernatants were clarified by centrifugation (1,500 g for 30 minutes) and filtration through a 0.8-µm filter (Sartorius, Göttingen, Germany). Viral stocks were evaluated for HIV-1 viral capsid protein (Gag p24) content using an ELISA kit (Organon Teknika, Fresnes, France). Single rounds of viral replication in P4 cells were performed as follows: P4 cells, seeded in 24-well plates (8 × 10⁴ cells per well), were infected the next day with 100 µL of HIV viral supernatant (100 ng of Gag p24). Virus and cells were left in contact for 8 hours at 37°C in the presence or absence of the different effectors, and the culture medium was then replaced with fresh medium containing 50 µM 3'-azido-3'-deoxythymidine (AZT; Sigma-Aldrich). Two days after infection, P4 cells were lysed and β-galactosidase (β-gal) activity was used as an index of HIV replication (42). Activated PBMCs (10⁶ cells) were infected with different HIV-1 isolates (100 ng of Gag p24) for 2 hours at 37° C with or without sPLA₂s (100 nM). Infected PBMCs were then cultured in the presence or absence of sPLA₂s (100 nM) in medium containing IL-2 (20 UI/mL) with fluid renewal each other day. Three and 6 days after infection, Gag p24 content in cell supernatants was determined as already indicated earlier here. The different inhibitors or catalytic products of PLA₂ activity were used in single-round infection assays and were preincubated with P4 cells in the presence or absence of sPLA₂s for 15 minutes before the addition of virus.

Effects of sPLA₂ on proviral DNA synthesis, syncytium formation, and cytosolic Gag p24 release. MT4 cells (10⁷ cells) were infected with HIV-1BRU (2.5 µg of Gag p24) in the absence or presence of effectors. Eight hours after virus addition, low–molecular-weight DNA was extracted from cells (43) and analyzed by Southern blot with an HIV-1BRU gag riboprobe (nucleotides 914–1920). For syncytium formation assay, P4 cells were mixed in a 3:1 ratio with 293 cells cotransfected 1 day before with pBru-2 and pCMVTat. Cells were cocultured in the presence of AZT (50 µM) and the different effectors for 48 hours before β-gal determination. Intracellular levels of cytoso-
lic Gag p24 were determined as described previously (44). Briefly, 10⁷ CEM cells were incubated at 4°C for 45 minutes with HIV-1BRU (1 µg of Gag p24); unbound viruses were then removed by 2 cold washes, and cells were incubated for 1 hour at 37°C in culture medium in the presence or absence of effectors. Infected cells were then treated with 50 µg/mL of pronase (Roche) for 5 minutes at 4°C to remove adherent viral particles. After extensive washes, cells were lysed by Dounce homogenization, and cytosolic fractions were prepared by ultracentrifugation at 150,000 g for 10 minutes at 4°C and used to determine the content of Gag p24.

sPLA₂ and gp120 binding assays. sPLA₂ binding experiments and cell-membrane preparations were performed as described using OS₂ as iodinated ligand (31). Briefly, membranes from P4 cells, ¹²⁵I-OS₂, and unlabeled competitors were incubated at 20°C in 1 mL of binding buffer (140 mM NaCl, 0.1 mM CaCl₂, 20 mM Tris [pH 7.4], and 0.1% BSA) for 90 minutes and then filtered through GF/C glass fiber filters (Whatman, Maidstone, United Kingdom) presoaked in 0.5% polyethylenimine. Filters were washed twice with binding buffer, and bound radioactivity was counted. gp120 (0.17 nmol) was labeled with ¹²⁵I- Na (0.5 nmol) to a specific activity of approximately 2,500 Ci/mmol using lactoperoxidase as described (45). ¹²⁵I-gp120 binding assays were performed on P4 cells that had been dissociated with PBS containing 2 mM EDTA, washed, and resuspended at 10⁷ cells/mL in gp120 binding buffer (50 mM HEPES [pH 7.2], 1 mM CaCl₂, 5 mM MgCl₂, and 0.5% BSA). Binding assays were performed in 150 µL of gp120 binding buffer containing P4 cells (10⁶ cells), ¹²⁵I-gp120 (3 x 10⁵ cpm, 0.5 nM), and various unlabeled competitors. After 30 minutes at 37°C, incubations were layered on 500 µL of PBS, centrifuged for 10 minutes at 20,000 g, and analyzed for radioactivity associated with cell pellets. Nonspecific ¹²⁵I-gp120 binding was determined in the presence of 300 nM unlabeled gp120.

### Table 1

<table>
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<td>21</td>
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</tr>
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</table>

*Activated human PBMCs were infected with the indicated primary HIV-1 isolates or laboratory adapted HIV-1 strains (100 ng of Gag p24). The amount of Gag p24 released into culture supernatant was measured after 3 and 6 days of culture in the continuous presence or absence of sPLA₂ (100 nM). *Cell viability was always greater than 80% in all conditions, as measured with the XTT assay. *Syncytium induction capacity in PBMCs.

**Results**

sPLA₂ have antiviral properties against T-tropic (X4 viruses) and M-tropic (R5 viruses) HIV-1 isolates. The effect of various sPLA₂ on HIV-1BRU infectivity was first analyzed with a single-round infection assay using CD4⁺ HeLa cells (P4 cells). As shown in Figure 1, 4 venom sPLA₂ (bvPLA₂, NmmCMIII, taipoxin, and nigexine) appear as very potent inhibitors of HIV-1 infection. These 4 sPLA₂ reduce HIV-1BRU replication with ID₅₀ values lower than 1 nM and completely prevent infection above 10 nM (Figure 1). In contrast, other venom sPLA₂ have weaker (OS₂, Biv) or no (PA2, PA5) antiviral activity (Figure 1). The inhibitory effect observed with Biv appears interesting, as Biv is a naturally occurring catalytically inactive sPLA₂ (41). We also analyzed the effect of human inflammatory-type group IIA sPLA₂ (hGIIA) and human and porcine pancreatic-type group IB sPLA₂ (hGIB and pGIB, respectively) (25). As shown in Figure 1, hGIIA, hGIB, and pGIB are very weak inhibitors of HIV-1BRU replication. Finally, we analyzed whether these sPLA₂ can interfere with the antiviral activity of bvPLA₂. In these experiments, P4 cells were infected with HIV-1BRU in the simultaneous presence of bvPLA₂ (10 nM) and either hGIB or hGIIA (up to 1 µM). In all cases, bvPLA₂ was found to retain full antiviral activity (data not shown), indicating that the inactive mammalian sPLA₂ do not interfere with the inhibitory activity of bvPLA₂.

Taipoxin, bvPLA₂, and NmmCMIII were then assayed for their ability to inhibit the replication of primary HIV-1 isolates and cell line–adapted HIV-1 strains that use either CXCR4 or CCR5 as a coreceptor. The replication of these HIV-1 isolates was measured in activated PBMCs maintained in the continuous presence or absence of sPLA₂ for 6 days (Table 1). HIV-1 replication, as monitored by the production of the HIV-1 Gag p24 protein, was dramatically reduced by sPLA₂ when PBMCs were infected either with a X4 virus (HIV-1BRU), a R5 virus (HIV-1ADA), or primary HIV-1 isolates of SI (91US054, CMU02) and NSI phenotype (91US056, 92US657). Similar antiviral activity of sPLA₂ was also observed when P4-CXCR5 cells were infected with the HIV-1 R5 viruses HIV-1ADA or HIV-1VYU (data not shown). Taken together, these data indicate that several, but not all, sPLA₂ protect efficiently different cellular models from the replication of HIV-1 isolates independently of the coreceptor usage.

sPLA₂ do not exert virucidal or cytotoxic effects and block virus entry. The lipid composition of the HIV viral membrane is similar to that of cell membranes and mainly contains glycerophospholipids, which are sPLA₂ substrates (46). A first possible mechanism for the inhibition of virus infection would then be that sPLA₂ inactivate viral particles by hydrolyzing the lipids of the viral membrane. To address this possibility, HIV-1BRU Viral particles were treat-
ed with bvPLA2 and then used to infect P4 cells (Table 2). Before infection, viral particles were centrifuged and the viral pellet was resuspended in the presence of specific bvPLA2 antibodies (rvAb) added to neutralize the residual bvPLA2 that contaminates the viral pellet. As shown in Table 2, HIV-1BRU viral particles treated with bvPLA2 were still able to infect P4 cells, clearly indicating that the antiviral activity of bvPLA2 does not result from a direct action on the viral particles.

Furthermore, pretreatment of target cells with bvPLA2 followed by extensive washes and infection with HIV-1 does not lead to inhibition of HIV-1 replication (Table 2). Another possibility would be that PLA2 exerts a cytoxic effect on the HIV-1 host cells. However, using the XTT cytotoxic assay, we found that the growth of the various HIV-1 host cells, including PBMCs (Table 1), was not affected by PLA2 treatment, indicating that the antiviral activity of PLA2s is different from a cytoxic effect (data not shown).

HIV-1 infection is initiated by the binding of gp120 to the CD4 receptor. This step was not blocked by PLA2s, as the binding of 125I-gp120 to CD4 was not inhibited by PLA2s including bvPLA2 (data not shown). This conclusion was confirmed by time-of-addition experiments showing that PLA2s are still able to inhibit HIV-1 replication when added to cells after virus binding (Figure 2a). In these experiments, P4 cells were incubated with HIV-1 at 4°C to allow virus binding, but not virus entry. Unbound viruses were then washed away, and cells were shifted to 37°C to trigger the virus-cell fusion process that initiates virus entry into host cells. At different times after virus addition to cells, bvPLA2 was added until virus entry was fully achieved (8 hours). As shown in Figure 2a, a complete inhibition of HIV-1 infection was observed when bvPLA2 was added before or after virus binding to cells. After the temperature shift, the sensitivity to bvPLA2 was gradually lost with an inhibitory half-time effect of about 2 hours. The inhibitory effect of bvPLA2 on virus entry occurs before the RT step, as the half-time effect of AZT, which is known to inhibit this step, was about 4 hours (Figure 2a). The half-time effects of taipoxin and NmmCMIII (data not shown) were found to be similar to those of bvPLA2, indicating that all of these PLA2s are acting after virus binding to cells, but before the RT step.

These results were confirmed by Southern blot analysis of the proviral DNA synthesis in HIV-1–infected cells (Figure 2b). For these experiments, MT4 cells were preferred because these cells are highly susceptible to HIV-1 infection and are also sensitive to PLA2 action (data not shown). Early after virus addition (8 hours), no unintegrated proviral DNA was detected in cells treated with bvPLA2, NmmCMIII, or taipoxin (Figure 2b), whereas it was abundantly accumulated in untreated cells and in cells treated with the mammalian PLA2s pGbIB (Figure 2b) and hGIIA (data not shown).

The fusion between viral and cellular membranes is usually addressed indirectly by syncytium formation assays between CD4+ cells and cells expressing gp120. The possible effect of PLA2s on the formation of syncytia was thus analyzed by cocultivation of P4 cells with 293 cells expressing HIV-1BRU proteins, including gp120. As shown in Figure 3a, bvPLA2 was unable to prevent the formation of syncytia, whereas both the chemokine SDF-1 (known to block virus-cell fusion by interacting with CXCR4; ref. 16) and an mAb specific for CD4 (Leu3A) led to a dramatic decrease in the formation of syncytia. These results indicate that the formation of syncytia between cells is not inhibited by PLA2s and suggest that the virus-cell fusion process is probably not affected by PLA2s.

Recently, Maréchal et al. have shown that the detection of Gag p24 in the cytosol of host cells can be used as a good index to monitor productive entry of HIV-1 into host cells (44). We therefore analyzed the effect of PLA2s on the level of cytosolic Gag p24 to determine whether PLA2s are inhibiting HIV-1 entry before or after virion dissociation from the cell membrane (Figure 3b). These experiments were carried out with different cell systems (P4, CEM, and MT4 cells), and suitable conditions were obtained with CEM cells. As shown in Figure 3b, CEM cells infected with HIV-1BRU in the presence of SDF-1, bvPLA2, taipoxin, or NmmCMIII contain much lower amounts of cytosolic Gag p24 compared with untreated cells or cells treated with hGIIA. Interestingly, PLA2s were as efficient as SDF-1 in preventing cytosolic Gag p24 accumulation (Fig-
Antiviral activity of sPLA₂ is linked to sPLA₂ binding to host cells, rather than sPLA₂ catalytic activity. The role of sPLA₂ catalytic activity in the HIV-1 antiviral action of sPLA₂s was addressed by using various sPLA₂ inhibitors that are known to inhibit sPLA₂ activity in vitro (23). Phenacyl bromide (0.1 mM), aristolochic acid (0.1 mM), and oleoyloxyethylphosphocholine (10 μM), which do not show antiviral activity by themselves, were unable to prevent the HIV-1 inhibitory effects of bvPLA₂ (data not shown). The cyclooxygenase blocker indomethacin (0.1 mM), which has no effect on HIV-1 infection, had also no effect on the antiviral activity of bvPLA₂ (data not shown). The effect of a neutralizing rabbit polyclonal serum raised against bvPLA₂ (rbvAb, 1:40), infection by measuring viral particles (HIV-1BRU) were incubated for 60 minutes at 37°C in the presence of sPLA₂s, such as arachidoninic acid, lysophosphatidylethanolamine, lysophosphatidic acid, and oleoyl-palmitoyllysophosphatidylcholine (up to 10 μM), were also found to be unable to block HIV-1 replication (data not shown). We also addressed the possible role of leukotriene B₄ (LTB₄), a downstream metabolite of PLA₂ activity whose receptor has recently been shown to act as a coreceptor for HIV-1 entry (47). Addition of LTB₄ (up to 1 μM) to P4 cells did not block HIV-1BRU replication (data not shown), making it unlikely that sPLA₂s mediate their inhibitory effect through production of LTB₄. Finally, the relatively weak but significant ability of the catalytically inactive sPLA₂ BaIV (41) to block HIV-1 replication (Figure 1) suggests that the sPLA₂ catalytic activity is not involved in HIV-1 inhibition. Together, these results indicate that the catalytic activity of sPLA₂s would not play a crucial role in the sPLA₂ antiviral effect.

Because sPLA₂s have previously been shown to bind specifically to membrane receptors (27), we analyzed the presence of sPLA₂ binding sites in the different cell systems used in this study. A specific sPLA₂ binding was observed in P4 cells (Figure 4, inset), as well as in PBMCs and CEM and MT4 cells (data not shown). Competition binding experiments (Figure 4) indicate that K₀.₅ values for bvPLA₂, taipoxin, nigexine, and NmcmβIII, which all block HIV-1 replication efficiently (Figure 1), are 0.08, 0.15, 0.06, and 0.17 nM, respectively (Figure 4 and data not shown). Conversely, K₀.₅ values for sPLA₂s that have weak or no antiviral activity (Figure 1) are in the micromolar range (OsI, BaIV) or higher (PA2, PA5, pGIB, hGIB, and hGIIA) (Figure 4 and data not shown). Taken together, the observed binding profile shows that the sPLA₂s that efficiently inhibit HIV-1 replication also display high affinities to P4 cell membranes, whereas the sPLA₂s that have no or weak inhibitory effect have much lower affinities.

Discussion

Mammalian and venom sPLA₂s have been implicated in a variety of physiological and pathological effects including cell proliferation, cell contraction, hormone release, inflammation, cancer, and antibacterial defense and exert various types of toxicities (22, 25, 30, 36, 48). We show here that several, but not all, sPLA₂s are potent inhibitors of HIV-1 replication. These sPLA₂s were found to protect different types of host cells efficiently from the replication of various HIV-1 isolates independently of the viral phenotype. Furthermore, bvPLA₂ was also able to inhibit the replication of HIV-2ROD10 in P4-CCR5 cells with ID₅₀ values close to 1 nM (data not shown), indicating that sPLA₂s can protect cells from infection with both HIV-1 and HIV-2 viruses.

Our results show that HIV inhibition by sPLA₂s takes place early during the viral life cycle. HIV enters the cell by fusion at the plasma membrane, a process that is triggered by the binding of gp120 to CD4 and chemokine receptors. Our data indicate that the binding of gp120 to these cellular receptors is not inhibited by sPLA₂s, because (a) binding of gp120 to CD4 is not inhibited by sPLA₂s; (b) sPLA₂s can block the entry of viruses that have been first bound to cells; (c) both X4 and R5 viruses that use different chemokine receptors are sensitive to sPLA₂s; and (d) syncytia formation between gp120 and CD4 expressing cells is not affected by the presence of sPLA₂s. The absence of sPLA₂ effect on syncytia formation also suggests that sPLA₂s do not act by blocking the virus-cell fusion process. However, this assumption must be tempered, as cell to cell fusion experiments do not always reflect the virus-cell fusion process (49).

Shortly after fusion, the disengagement of the RT complex from the cell membrane (often referred as uncoating) leads to the cytosolic accumulation of virion components. This step is believed to be a prerequisite for the synthesis of the proviral DNA that then moves toward the cell nucleus (10, 13, 14, 50). Our data show that sPLA₂s do not act directly on viral particles (Table 2), do not act on cells before infection (Table 2), do not block CD4-gp120 interaction, and have no antiviral activity when added a few hours after virus addition (Fig-

### Table 2

<table>
<thead>
<tr>
<th>Pretreatment of viral particles</th>
<th>Pretreatment of cells</th>
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<th>Viral replication (%)</th>
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</table>

*Viral particles (HIV-1BRU) were incubated for 60 minutes at 37°C in the presence or absence of bvPLA₂ (10 nM), ultracentrifuged, and then used to infect P4 cells. ²P4 cells were incubated for 2 hours in medium containing or not containing bvPLA₂ (10 nM), washed, and infected with HIV-1BRU. ³P4 cells were infected with HIV-1 viral particles in medium containing bvPLA₂ (10 nM) or a neutralizing rabbit polyclonal serum raised against bvPLA₂ (rbV₀₁, 1:40), as indicated. ⁴The level of viral replication was determined 48 hours after infection by measuring β-gal activity. Each value represents the mean of at least 3 independent experiments with an SEM less than 10%.
Binding properties of sPLA₂s to P4 cell membranes. P4 cell membranes (20 μg/mL) were incubated with iodinated sPLA₂ (25 pM) and various concentrations of unlabeled sPLA₂s. Results are expressed as percentage of the maximal specific binding measured in the absence of sPLA₂ competitor. A total of 100% corresponded to 1.8 pM of specifically bound labeled sPLA₂. Nonspecific binding was measured with 100 nM competitor. A total of 100% corresponded to 1.8 pM of specifically bound labeled sPLA₂. Nonspecific binding was measured with 100 nM unlabeled ligand and accounted for 10% of total binding.

**Figure 4**

Binding properties of sPLA₂s to P4 cell membranes. P4 cell membranes (20 μg/mL) were incubated with iodinated sPLA₂ (25 pM) and various concentrations of unlabeled sPLA₂s. Results are expressed as percent binding of the maximal specific binding measured in the absence of sPLA₂ competitor. A total of 100% corresponded to 1.8 pM of specifically bound labeled sPLA₂. Nonspecific binding was measured with 100 nM competitor. A total of 100% corresponded to 1.8 pM of specifically bound labeled sPLA₂. Nonspecific binding was measured with 100 nM unlabeled ligand and accounted for 10% of total binding.

We thus conclude that sPLA₂s display antiviral activity only when they are present during the first hours of virus entry that correspond to the critical period of sPLA₂ action. This is in agreement with our results showing that sPLA₂s prevent the release of Gag p24 and the synthesis of proviral DNA in the cytosol of cells infected with HIV-1 (Figures 2 and 3), and suggests that sPLA₂s block virus entry by preventing virion uncoating and dissociation of the RT complex from host cell membranes. This blockade of virion disassembly is likely to explain the lack of proviral DNA synthesis in the presence of sPLA₂s, as these enzymes were unable to inhibit the RT activity (data not shown), as measured in both exogenous and endogenous in vitro assays (51). Several previous reports have shown that postfusion steps can be modulated by different cellular and viral factors. The phosphorylation of RT complex components by a virion-associated kinase facilitates the disengagement of this complex from the cell membrane and its subsequent nuclear targeting (12, 14, 52, 53). Cyclophilin A, a host protein isomerase, is required for an early step in the HIV-1 life cycle, probably to favor virion uncoating by destabilizing the capsid structure (54, 55). Nef, a regulatory viral protein found associated to the virion (56), increases the efficiency of RT (51, 57) and may increase viral nuclear import (58). It thus appears that postfusion steps are tightly regulated, making it possible that sPLA₂s act on one of these steps through direct or indirect interaction with viral or cellular factors.

The sPLA₂ catalytic activity does not appear to be involved in HIV-1 antiviral effect. First, different sPLA₂ inhibitors were unable to prevent sPLA₂ antiviral activity. Second, sPLA₂ products, such as arachidonic acid and various lysophospholipids, do not prevent HIV-1 replication. Third, several sPLA₂s like PA2, PA5, pGIB, hGIB, and hGIIA are catalytically active enzymes (25, 27), but do not have antiviral activities (Figure 1). Finally, BaIV, a catalytically inactive sPLA₂-like protein (41), was found to have antiviral properties, although with a relatively low ID₅₀ value of 400 nM (Figure 1). On the other hand, the sPLA₂ antiviral activity appears to be associated to the ability of sPLA₂s to bind to host cells. Indeed, the Kₐ₅ values measured for the binding of the various sPLA₂s including BaIV to P4 cells are in good agreement with their ID₅₀ values to inhibit HIV-1 infection (Figures 1 and 4). So far, 2 main types of sPLA₂ receptors have been identified (27). N-type receptors display high affinities for bvPLA₂, taipoxin, and NmmCMIII sPLA₂, and low affinities for OS₁ and pGIB. Conversely, M-type receptors have high affinity for OS₁ and pGIB, and do not bind bvPLA₂ and NmmCMIII. Based on these binding profiles, the sPLA₂ binding sites detected in P4 cells appear most similar to N-type receptors. Furthermore, no M-type receptors were detected in P4 cells (data not shown). The molecular nature of the sPLA₂ binding sites found in P4 cells remains to be determined, but are likely to be related to the N-type like receptors that have been identified in different tissues and cells including immune cells (27, 31, 32).

In conclusion, our data indicate that several venom sPLA₂s can protect various types of host cells including PBMCs from the replication of primary HIV-1 isolates. Conversely, human group IB and human group IIA sPLA₂s were unable to block HIV-1 replication. However, because several other sPLA₂s are being identified in mammals (26, 28, 29), it is tempting to speculate that some of these novel human sPLA₂s may have clinical relevance in HIV infection. In individuals repeatedly exposed to HIV but who remain uninfected, several possible reasons for protection have been proposed but not clearly elucidated (59). In this respect, the putative antiviral activity of the novel group IIB, V, and X sPLA₂s, which are expressed in immune tissues and cells such as macrophages and PBMCs (28, 29), will be particularly interesting to analyze in the future, when these enzymes will be available in high enough amounts to assay them, as we have assayed venom sPLA₂.

**Acknowledgments**

We thank P. Rochet V. Sansoldi, and N. Gomez for expert technical assistance. The authors are grateful to P. Charneau, F. Clavel, K. Peden, N. Israel, and the NIH-AIDS Research and Reference Reagent Program for the generous gifts of the various plasmids and cell lines. We are also indebted to A. Ménez, J. Christophe, H. Verheij, J.M. Gutiérrez, and S. Lizano for providing the various plasmids and cell lines. The authors acknowledge the generous gifts of the various plasmids and cell lines. The authors are grateful to P. Rochet V. Sansoldi, and N. Gomez for expert technical assistance. The authors are grateful to P. Charneau, F. Clavel, K. Peden, N. Israel, and the NIH-AIDS Research and Reference Reagent Program for the generous gifts of the various plasmids and cell lines. We are also indebted to A. Ménez, J. Christophe, H. Verheij, J.M. Gutiérrez, and S. Lizano for providing the various plasmids and cell lines. E. Valentin is a recipient of a grant from the region Provence Alpes Côte d’azur–CNRS program.


