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Phosphatidylserine on HIV Envelope Is a Cofactor for Infection of Monocytic Cells[1]

Melissa K. Callahan,[2,3]* Paul M. Popernack,[2†] Shigeki Tsutsui,[4‡] Linh Truong,[‡] Robert A. Schlegel,*[§] and Andrew J. Henderson[5]*†‡

HIV-1 is an enveloped retrovirus that acquires its outer membrane as the virion exits the cell. Because of the association of apoptosis with the progression of AIDS, HIV-1-infected T cells or macrophages might be expected to express elevated levels of surface phosphatidylserine (PS), a hallmark of programmed cell death. Virions produced by these cells would also be predicted to have PS on the surface of their envelopes. In this study, data are presented that support this hypothesis and suggest that PS is required for macrophage infection. The PS-specific protein annexin V was used to enrich for virus particles and to inhibit HIV-1 replication in primary macrophages, but not T cells. HIV-1 replication was also significantly inhibited with vesicles consisting of PS, but not phosphatidylcholine. PS is specifically required for HIV-1 infection because viruses pseudotyped with vesicular stomatitis virus G and amphotropic murine leukemia virus envelopes were not inhibited by PS vesicles or annexin V. These data indicate that PS is an important cofactor for HIV-1 infection of macrophages. ***The Journal of Immunology,*** **2003, 170: 4840–4845.**

Human immunodeficiency virus is an enveloped retrovirus whose entry into permissive target cells is dependent on receptor-mediated fusion between viral and target cell membranes. Binding and fusion are mediated by viral envelope proteins gp120, which interacts with host CD4 and chemokine receptors, and gp41, which contains an N-terminal fusion peptide. Recent attention has focused on interactions between gp120 and chemokine receptors, especially CXCR4 and CCR5, providing insight into factors that influence tropism of HIV-1, the progression of AIDS, and potential novel treatment strategies (1). Although CD4, chemokines, and their receptors are clearly important in the establishment of HIV infection, there is increasing evidence that additional surface molecules act as cofactors for binding and entry. Reports of primary HIV isolates that infect cell lines lacking CD4 and chemokine receptors suggest that there are uncharacterized accessory molecules that participate in infection (2–5). In addition, HIV envelope, which is acquired as the virus particle exits the cell, includes proteins and lipids that are selectively obtained from the host membrane. The inclusion of host-derived proteins such as ICAM-1 and MHC-II molecules has been demonstrated to enhance infection possibly by binding counterreceptors on target cells and stabilizing virus-cell interactions (6–10).

Although much attention has focused on how specific proteins mediate virus-cell fusion, relatively little is known about the role lipids have in this process. The presence of different lipids in either target cell or virus membranes can dramatically influence infection. For example, glycosphingolipids in target cell membranes have been shown to increase HIV infection (3, 11–13). Furthermore, the viral envelope, despite being acquired from host cells, has a lipid composition that differs from the host plasma membrane with elevated levels of sphingolipids, cholesterol, and a subset of phospholipid species (14).

One enriched membrane phospholipid in the HIV envelope is phosphatidylserine (PS)[6] (14). PS is normally sequestered to the inner leaflet of the plasma membrane bilayer. However, during the course of apoptosis, the mechanism that normally maintains PS in the inner leaflet is down-regulated (15), allowing the appearance of PS on the cell surface. PS exposure is a hallmark of apoptosis and a recognition signal for phagocytic cells that clear dying cells (16, 17). Several macrophage receptors have been implicated in recognizing PS on apoptotic cells, including various scavenger receptors, CD36, CD14, and PS receptor (PSR) (16). Thus, PS has a demonstrated ability to mediate cell-cell interactions and to function as a ligand, making its appearance in the viral membrane highly suspect as a factor in virus-target cell fusion.

Because the primary targets of HIV infection are $CD4^+$ T cells and macrophages, the virus envelope will acquire specific properties of these cellular plasma membranes. Following HIV-1 infection, T cells are highly susceptible to programmed cell death, and peak levels of T cell apoptosis correlate with high levels of virus replication (18). Furthermore, mature macrophages constitutively express PS in the outer leaflet of their plasma membranes (19), although their precursors, monocytes, do not (20). Therefore, HIV-infected T cells and macrophages would be expected to have elevated levels of surface PS, and HIV-1 particles produced by these cells would have PS incorporated into the outer leaflet of their envelopes. We directly tested this hypothesis and demonstrated

Graduate Programs in *Biochemistry, Microbiology, and Molecular Biology and [†]Pathobiology, and Departments of [‡]Veterinary Science and [§]Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA 16802

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[2] M.K.C. and P.M.P. contributed equally to this work.

[3] Current address: Cleveland Clinic Foundation, Neuroscience Dept. NC3-106, 9500 Euclid Avenue, Cleveland, OH 44195.

[4] Current address: Department of Clinical Neurosciences, University of Calgary, 3330 Hospital Drive, NW, Calgary, AB T2N 4N1, Canada.

[5] Address correspondence and reprint requests to Dr. Andrew J. Henderson, Department of Veterinary Science, Immunology Research Laboratories, Pennsylvania State University, 115 Henning Building, University Park, PA 16802. E-mail address: ajh6@psu.edu

[6] Abbreviations used in this paper: PS, phosphatidylserine; aMLV, amphotropic murine leukemia virus; MDM, monocyte-derived macrophage; PC, phosphatidylcholine; PLAP, placental alkaline phosphatase; PSR, PS receptor; RT, reverse transcriptase; VSV-G, vesicular stomatitis virus glycoprotein.

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that PS is present on the surface of the HIV-1 envelope. More importantly, our results suggest that this phospholipid is a cofactor for macrophage infection.

Materials and Methods

Cells

U937 and THP-1 monocytic cells and Jurkat $CD4^+$ T cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine and 10% FBS at 37°C in 5% CO_2. The 293T cells were cultured in DMEM supplemented with 10% FBS and incubated at 37°C in 5% CO_2.

Mononuclear cells were obtained by differential centrifugation using a Ficoll/Hypaque gradient (Sigma-Aldrich, St. Louis, MO), as previously described (21). Monocyte-derived macrophages (MDM) were separated from lymphocytes by adherence to plastic overnight and allowed to differentiate in vitro for 5–7 days before infection. $CD4^+$ T cells were purified from nonadherent mononuclear cell fraction using Dynal microbeads coated with anti-human CD4 Abs and magnetic separation (Dynal, Lake Success, NY), following manufacturer's protocol.

Generation of HIV-1 infectious titers and infections

Replication-competent HXB.2, Ba-L, and HXBnPLAP-IRES-N^+ (22) viruses were generated by transfecting 293T cells with 15 μg of DNA viral clones and 3 μg of Rous sarcoma virus-Rev DNA by calcium phosphate transfection (23). Infectious virus at a multiplicity of infection of 0.5–0.1 was added to cells in the absence or presence of annexin V, PS, or phosphatidylcholine (PC) vesicles at the indicated concentrations. The infection medium was removed 24 h postinfection and replaced with fresh medium with or without annexin V or phospholipid vesicles. For some experiments, the culture medium was also supplemented with 2 mM $CaCl_2$. Virus replication was monitored by measuring reverse transcriptase (RT), as described previously (21), or by p24 ELISA (NEN, Boston, MA). Sucrose density gradient-purified HIV-1 IIIB passed through H9 T cells was obtained from Advanced Biotechnologies (Columbia, MD).

Vesicular stomatitis virus glycoprotein (VSV-G) and amphotropic murine leukemia virus (aMLV)-pseudotyped HIV-1 was generated by transient transfection using 15 μg of pNL43-Luc^+Env^- DNA (24), 3 μg of pLTR(L)-VSV-G DNA (25), and 3 μg of Rous sarcoma virus-Rev DNA. Infections were performed, as described above. Cells were harvested 48 h postinfection for luciferase assays using the Promega luciferase assay system (Promega, Madison, WI).

Preparation of annexin V and phospholipid vesicles

Annexin V was produced and affinity purified from a bacterial expression system, as previously described (26, 27). A single band that migrated at ~30 kDa was observed on a silver-stained 12% SDS-polyacrylamide gel following purification.

To prepare lipid vesicles, bovine brain PS and egg PC (Sigma-Aldrich) were dried under nitrogen, resuspended in PBS by vortexing, and sonicated for 10 min. Vesicles were used at final concentrations spanning 15 nM to 1 μM (28).

Magnetic separation of virions and HIV-1-infected cells

The Dynal magnetic separation system (Dynal) was used to enrich for virions and HIV-1-infected cells. Annexin V-coated Dynabeads were generated by incubating streptavidin-conjugated beads with biotinylated annexin V. Annexin V-coated beads were added to various concentrations of virus stock, supplemented with 2 mM $CaCl_2$ or 0.5 mM EGTA, and incubated for 30 min at room temperature. Beads were collected with a magnet, and associated virus was monitored by RT activity or p24 ELISA.

Placental alkaline phosphatase (PLAP) expression was used as a positive marker for cells infected with HXBnPLAP-IRES-N^+ virus (22). $PLAP^+$ cells were isolated using CELLection Pan Mouse IgG kit from Dynal, following manufacturer's protocols. Pan mouse IgG Dynabeads were coated with mouse anti-PLAP Abs (Sigma-Aldrich). Cells infected with HXBnPLAP-IRES-N^+ were incubated with beads at 4°C for 15 min in RPMI supplemented with 1% FBS. A magnet was used to enrich for $PLAP^+$ cells, which were released from beads by incubating with Dynal releasing buffer. Expression of PLAP was confirmed by flow cytometry (data not shown).

Flow cytometry

Cells were stained for PLAP expression using the primary Ab mouse anti-human placental alkaline phosphatase (Sigma-Aldrich) and the secondary Ab FITC-conjugated rat anti-mouse IgG2a (clone R19-15; BD PharMingen, San Diego, CA). Cells stained with secondary Ab alone were used as a negative control. For annexin V staining, cells were resuspended in annexin V staining buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) before staining with PE-conjugated annexin V (BD PharMingen). Propidium iodide was added before flow cytometry to gate out dead cells (19). As negative controls for all annexin V staining, cells were extensively washed in Ca^{2+}-free PBS before adding annexin V.

Cells were analyzed using an EPICS-XL-MCL flow cytometer (Coulter Electronics, Hialeah, FL) fitted with a single 15-mW argon ion laser providing excitation at 488 nm. FITC was monitored through a 525-nm bandpass filter, whereas PE was detected through a 575-nm bandpass filter.

Virus-binding assay

HIV-1 was radiolabeled by adding 1 mCi of ^{35}S-labeled L-methionine (>1000 Ci/mM; ICN Biomedicals, Irvine, CA) to culture medium following 293T transfection. Labeled virions at a multiplicity of infection of 1–10 were incubated with 1×10^6 cells/ml U937, THP-1, and MDM in the presence or absence of anti-CD4 Ab, soluble CD4 (obtained from National Institutes of Health AIDS Research and Reference Reagent Program), annexin V, PS, or PC vesicles at the indicated concentrations for ~3 h at room temperature. Cells were collected by centrifugation, washed with PBS three times before detecting cell-associated virus by measuring cpm with a Beckman LS 6000IC scintillation counter.

Results

HIV-1 virion has external PS

HIV-1 infection is associated with increased apoptosis. A hallmark of apoptotic cells is surface expression of PS, a phospholipid that is normally restricted to the inner leaflet of the plasma membrane (16). If cells infected with HIV-1 undergo apoptosis, they would be expected to express PS on their surface. We therefore examined various HIV-1-infected cells for surface PS. To identify and positively select for infected cells, cells were infected with a replication-competent clone that carries the reporter gene PLAP (22), and cells expressing PLAP were separated using magnetic beads. HIV-1-positive and HIV-1-negative populations were then examined for surface PS by staining with fluorescent annexin V, which binds specifically to PS. A total of 61% of Jurkat T cells and greater than 85% of either U937 or THP-1 monocytic cells that were PLAP positive also stained positively with annexin V, whereas fewer than 15% of the uninfected cells in the same population had surface PS (Fig. 1). Greater than 90% of the cells were annexin V negative before infection (data not shown). Increased surface PS was also observed on U1 cells, which harbor inducible latent provirus, following activation of virus expression with phorbol ester (Fig. 1), and on 293T cells after transient transfection of HIV-1 cDNA expression constructs (data not shown). These data are consistent with previous reports that showed a correlation with HIV-1 replication and annexin V-positive staining cells (18).

Because HIV-1 acquires its envelope upon exiting the cell, it would be expected that the virion would also have PS in the external leaflet of its membrane. This was tested by determining whether beads coated with annexin V could specifically bind and pull down HIV-1. Initially, we used HXB.2, which expresses CXCR4-tropic (X4) envelope, that was packaged in 293T cells. In the presence of Ca^{2+}, which is required for PS-specific binding, annexin V-coated beads bound significant levels of HIV-1, as determined by RT activity or associated p24 levels (Fig. 2). If Ca^{2+} was depleted with EGTA or uncoated beads were used, no significant HIV-1 binding was detected (Fig. 2). To minimize cell membrane contamination and assure that external PS on the virion was not an artifact of packaging virus in 293T cells, we used sucrose gradient-purified IIIB virus produced by H9 T cells. Consistent with the above results, annexin V-coated beads pulled down HIV from this source (Fig. 2*B*). These data indicate that HIV-1 has PS exposed on its envelope surface.

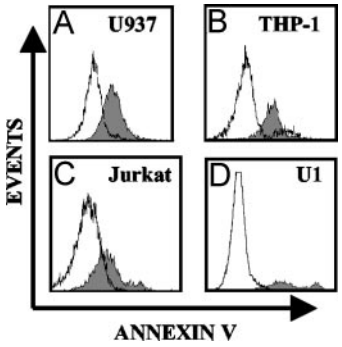

FIGURE 1. HIV-1-producing cells have external PS. U937 (*A*), THP-1 (*B*), and Jurkat (*C*) cells were infected with HXBnPLAP-IRES-N$^+$ for 6–9 days. PLAP-expressing cells were positively selected using Dynal magnetic separation, as described in *Materials and Methods*, and stained for PS using annexin V (filled histogram). Cell populations depleted of HIV-1-infected cells were also stained for annexin V (open histogram). *D*, U1 cells untreated (open histogram) or stimulated with PMA (filled histogram) were stained with annexin V. For these experiments, propidium iodide was added before flow cytometry to gate out dead cells. Virus expression was confirmed in these experiments by measuring RT activity (data not shown). Histograms for unstained cells, uninfected cells, or cells stained in the absence of Ca^{2+} showed no significant staining and completely overlapped the profiles for the cell populations depleted of HIV-1-infected cells (data not shown).

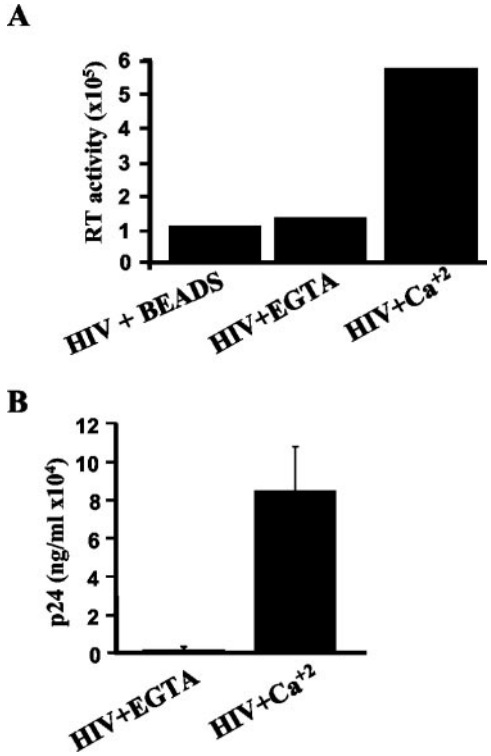

FIGURE 2. HIV-1 virions have PS in the outer leaflet of the envelope. *A*, A total of 1×10^6 virions, prepared from transfected 293T cells, as described in the *Materials and Methods*, were added to uncoated streptavidin-conjugated beads or annexin V-coated beads in the presence of 2 mM $CaCl_2$ or 0.5 mM EGTA and incubated for 30 min. Beads were collected with a magnet, and associated virus was determined by RT activity. These data are from a single experiment that represents three independent experiments that gave similar results. *B*, A total of 1×10^7 HIV virions prepared from H9 T cells and concentrated by sucrose gradient were added to annexin V-coated beads, as described above. Virus associated with the beads was determined by p24 assays. Background binding, as determined with streptavidin-conjugated beads, was subtracted from the raw values. These data represent three independent experiments, and the error bars represent a single SD.

PS enhances HIV-1 infection of macrophages

Annexin V was used to block PS on the virion surface to determine whether this phospholipid had a role in HIV-1 infection. Jurkat T cells and U937 and THP-1 monocytic cell lines were infected with HXB.2 X4 virus in the absence or presence of annexin V. The presence of annexin V had no significant effect on the infection of Jurkat T cells (Fig. 3*A*). However, an 8-fold decrease in virus production was observed when U937 and THP-1 cells were infected in the presence of annexin V (Fig. 3). Infection with sucrose gradient-purified IIIB was also inhibited by annexin V treatment (data not shown). The ability of annexin V to inhibit the infection of monocytic cells was dose dependent (Fig. 3*B* and data not shown). Furthermore, expression of a replication-incompetent HIV-luciferase clone that was pseudotyped with HXB.2 envelope was inhibited by 80% in the monocytic cell lines when infected in the presence of annexin V (Fig. 6*C*). Similar results were observed using primary CD4$^+$ T cells and MDM. As shown in Fig. 4, annexin V did not alter HIV-1 infection of primary CD4$^+$ T cells, although it significantly inhibited infection of MDM by CCR5 (R5) tropic viruses. These latter results demonstrate that this phenomenon is not restricted to monocytic cell lines nor associated with specific HIV envelopes. In addition, HIV-1 infection of monocytic cell lines and MDMs was blocked 60–80% by artificial lipid vesicles composed of PS, whereas vesicles composed of phosphatidylcholine (PC) had no significant effect on HIV-1 expression (Fig. 5). These results demonstrate that PS is an important cofactor for HIV-1 infection of monocytes/macrophages.

The ability of PS to influence HIV-1 infection depends on HIV-1 envelope proteins

To examine whether annexin V and PS vesicles specifically inhibited gp120-mediated infection or whether these reagents resulted in a more general inhibition of viral entry, the replication-defective HIV-luciferase virus was pseudotyped with either VSV-G, which permits entry by a receptor-mediated endocytic pathway, or aMLV envelope, which uses the sodium-dependent phosphate symporter Pit-2 for entry (29, 30). These viruses were used to infect various cell lines and primary macrophages, and proviral expression was monitored by luciferase activity. Despite evidence that PS was on the surface of the different pseudotyped viruses (data not shown), annexin V or PS vesicles did not significantly inhibit VSV-G-HIV-1 or aMLV-HIV-1 expression, whereas infection with HXB.2 X4 envelope was strongly compromised (Fig. 6 and data not shown). These data suggest that PS is a specific cofactor for HIV-1 entry into monocytic cells.

Blocking PS does not alter HIV-1 binding

To gain insight into the mechanism by which PS influences HIV-1 infection, we directly assessed whether annexin V or PS vesicles affected HIV-1 binding to target cells. Infectious virus stocks were radiolabeled by packaging in the presence of [^{35}S]methionine. Following incubation with virus for 3 h and several washes, cells were collected and associated virus was detected by measuring ^{35}S. Significant HIV-1 binding to U937 monocytes was observed regardless of whether annexin V or PS vesicles were present, whereas soluble CD4 significantly blocked HIV-1 binding (Fig. 7). Similar results were observed in experiments with THP-1 cells and primary MDM (data not shown). Therefore, although PS on the surface of HIV-1 envelope enhances HIV-1 infection of macrophages, it does not influence binding of the virus to the target cell.

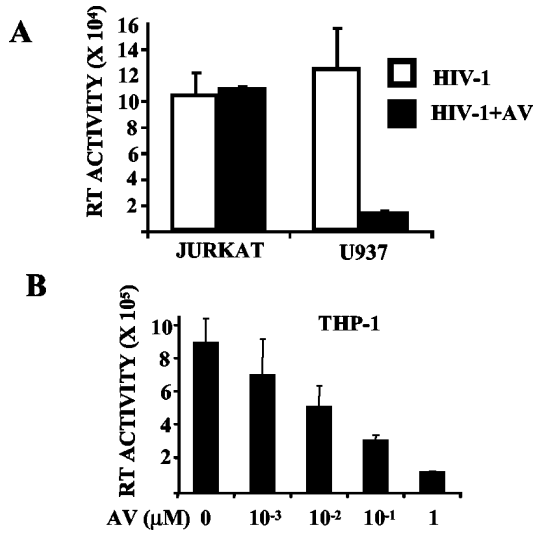

FIGURE 3. Annexin V inhibits infection of monocytes/macrophages. *A*, Jurkat T cells and U937 monocytic cells were infected with HXB.2 in the presence or absence of 0.1 μM annexin V. Virus replication was monitored by RT assays 6 days postinfection. *B*, THP-1 monocytic cell line was infected with HXB.2 in the presence of varying concentrations of annexin V. Virus replication was monitored on day 8 postinfection.

Discussion

The interaction between HIV-1 and its target cells is complex and not explained entirely by the presence or absence of CD4 and chemokine receptors such as CXCR4 and CCR5 (2, 31). Several molecules, including ICAM-1, LFA-1, MHCII, CD28, glycosaminoglycans, and glycosphingolipids, on the surface of the cell and virus have been shown to participate in HIV-1 infection, possibly by stabilizing virus-cell interactions or promoting postbinding events (3–6, 8, 9, 11–13, 32). Furthermore, HIV-1 infection independent of CD4 and chemokine receptors has been described, suggesting that novel receptors may be expressed on different cell types (2).

Little attention has been devoted to the lipid composition of the HIV-1 envelope or the potential importance that lipids have in the infection process. Anionic phospholipids, including PS, have been implicated as receptors and cofactors for several viruses, including VSV, rhabdoviruses, hepatitis B, influenza viruses, hemorrhagic septicemia, Sendai virus, rubella virus, and Sindbus virus (33–40). Characterization of lipid composition of HIV-1 envelope shows

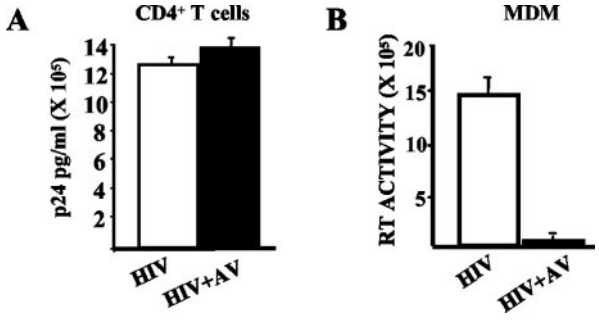

FIGURE 4. Annexin V inhibits infection of primary macrophages. *A*, $CD4^+$ T cells were infected with HXB.2 in the presence or absence of 0.1 μM annexin V. Virus replication was monitored at day 6 by p24 ELISA. *B*, MDMs were infected with HIV Ba-L in the absence or presence of annexin V, and RT activity was determined 9 days postinfection. Each bar represents three independent infections. These data are single experiments that have been repeated at least three times. Error bars represent a single SD.

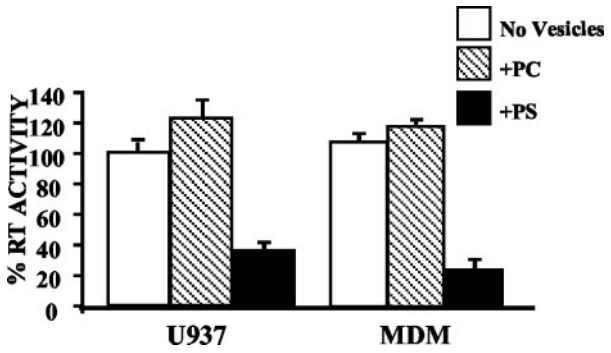

FIGURE 5. PS vesicles inhibit HIV-1 infection. U937 and MDM were infected with either HXB.2 or Ba-L, respectively, in the absence or presence of 15 nM PS or PC vesicles. Six days postinfection, virus replication was monitored by RT assays. Data are shown as percentage of RT activity in untreated infected cells. Each data point is derived from three independent infections, and the error bars represent a single SD. These data are from single experiments that represent three independent experiments that gave similar results.

that many lipids, including glycosphingolipids, cholesterol, and PS, are found in the membrane at a higher frequency than what is represented in the host membrane, suggesting a selective incorporation of these lipids (14). It has been demonstrated that glycosphingolipids on the surface of the cell enhance HIV-1 infection. Furthermore, HIV-1 envelope requires cholesterol for membrane organization and function (41–43). Using model liposomes, HIV-1 fusion has been shown to be dependent on lipid composition, and

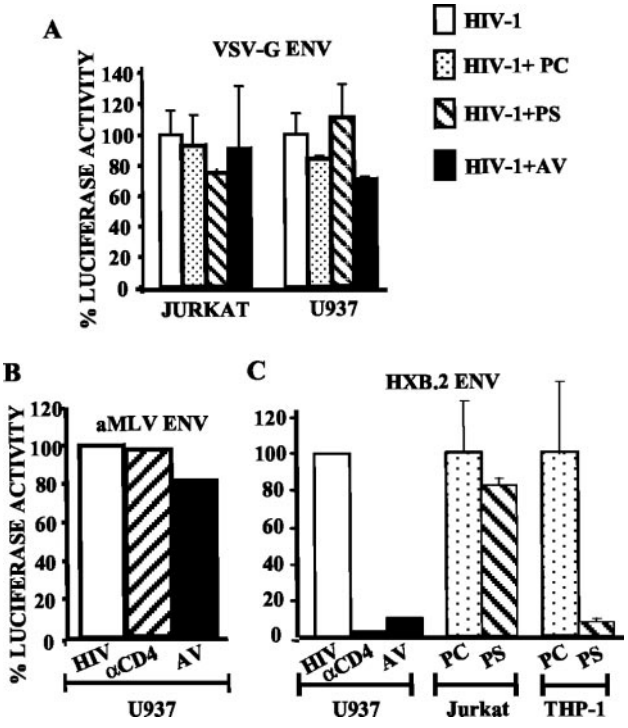

FIGURE 6. gp120 is required for inhibition of HIV-1 infection by annexin V. Replication-incompetent HIV-luciferase pseudotyped with VSV-G (*A*), aMLV (*B*), and HXB.2 envelopes (*C*) was used to infect the indicated cells in the absence or presence of 1 μg anti-CD4 Ab, 0.1 μM annexin V, 15 nM PS vesicles, or PC vesicles. Forty-eight hours postinfection, HIV-1 infection was determined by measuring luciferase activity. Each bar represents at least two independent infections. Error bars show 1 SD. These data are from a single experiment that has been performed at least three times.

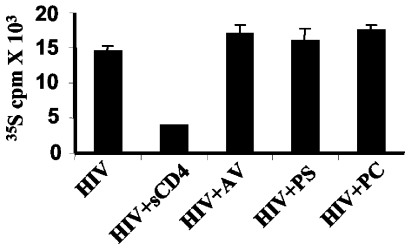

FIGURE 7. Annexin V and PS vesicles do not inhibit HIV-1 binding. U937 cells were cocultured with ^{35}S-labeled virus for 3 h in the absence or presence of 5 μg of sCD4, 1 μM annexin V, 15 nM PS vesicles, or 15 nM PC vesicles. Cells were collected by centrifugation and washed three times, and bound virus was determined by measuring cpm in the cell pellet. Each data point represents at least three independent infections. These data are from a single experiment that has been performed three times.

cardiolipin vesicles inhibit infection (44, 45). Our results would add PS to this short list of critical lipids that influence HIV-1 infection.

We demonstrate that PS is in the outer leaflet of the HIV-1 membrane, and if this lipid is blocked by annexin V or competed for by excess PS, infection of monocytic cells is significantly inhibited. Because HIV-1 acquires its envelope from the host cell, the observation that PS is in the outer leaflet of the virus membrane is consistent with studies that show a positive correlation between HIV-1 replication and apoptosis (18). Annexin V is most likely inhibiting infection by blocking PS on the envelope, rather than the target cells, because cells do not positively stain with annexin V before infection. That the effect is restricted to monocytic cells and does not extend to T cells implicates a PSR(s) on macrophages. Macrophages express an array of receptors that recognize PS, including members of the scavenger receptor family, β integrins, PSR, and CD14 (reviewed in Refs. 16, 46, and 47), although whether any of these receptors participate in HIV-1 infection has not been investigated. Preliminary studies suggest a limited role for CD14 in HIV-1 infection of macrophages because Abs that block CD14 did not adversely affect virus entry (data not shown).

The results presented in this study suggest that recognition of PS by macrophage receptors is not required for virus binding. However, by engaging specific receptors on macrophages, PS could stabilize virus-cell interaction and contribute to more efficient fusion. It is also possible that viral-associated PS engaging a specific receptor or complex of receptors initiates signaling cascades that activate host cell processes, such as cytoskeletal rearrangements, necessary for virus entry. This would be consistent with a recent model proposed for phagocytosis of apoptotic cells, which suggests that upon engagement with PS, the PSR transduces signals required for activating the phagocytosis machinery (46, 48, 49).

PS, by virtue of its physical properties, may contribute to a more fusion-competent virion. PS in the outer leaflet of membranes has been implicated in mediating cell fusion, exocytosis, and signal transduction (50–52). Studies with erythrocytes have shown that fatty acyl side chains of PS are less saturated than the side chains of other phospholipids, and that loss of membrane asymmetry increases membrane fluidity and surface hydrophobicity (50). Therefore, by altering membrane structure and organization, PS in the outer bilayer of the HIV-1 envelope may enhance virus fusion and entry.

The above models assume that PS is acting by influencing virus entry; however, annexin V or PS vesicles do not dramatically influence HIV-1 attachment to target cells, suggesting that it is unlikely that PSRs are serving as major coreceptors for HIV-1. It has been suggested that at least one PSR on macrophages is not responsible for binding, but rather for signal transduction (48). Furthermore, engulfment of apoptotic cells, which requires external PS, alters cytokine expression and macrophage function (51–54). Therefore, it is possible that recognition of virus-associated PS by macrophages could directly affect reverse transcription and/or proviral transcription or indirectly influence HIV-1 replication by altering the cytokine microenvironment. Although we have not seen any effects on HIV-1 transcription in infected monocytes/macrophages treated with annexin V or PS vesicles, we cannot rule out that PS recognized in the context of a virus particle or apoptotic cell alters HIV-1 expression (54, 55). Current studies are focusing on mechanisms by which PS influences HIV-1 replication and at what stages in the viral life cycle this lipid is acting.

In summary, our data demonstrate that PS in the outer leaflet of the HIV-1 virion is critical for HIV-1 fusion and entry into macrophages. In addition to identifying a lipid cofactor that influences HIV-1 infection and novel targets for blocking HIV-1 entry, these studies suggest that general properties of virus membranes impact infection, tropism, and the course of AIDS.

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