PI4P5-Kinase Iα Is Required for Efficient HIV-1 Entry and Infection of T Cells[1]

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HIV-1 envelope (Env) triggers membrane fusion between the virus and the target cell. The cellular mechanism underlying this process is not well known. Phosphatidylinositol 4,5-bisphosphate (PIP_2) is known to be important for the late steps of the HIV-1 infection cycle by promoting Gag localization to the plasma membrane during viral assembly, but it has not been implicated in early stages of HIV-1 membrane-related events. In this study, we show that binding of the initial HIV-1 Env-gp120 protein induces PIP_2 production in permissive lymphocytes through the activation of phosphatidylinositol-4-phosphate 5-kinase (PI4P5-K) Iα. Overexpression of wild-type PI4P5-K Iα increased HIV-1 Env-mediated PIP_2 production and enhanced viral replication in primary lymphocytes and CEM T cells, whereas PIP_2 production and HIV-1 infection were both severely reduced in cells overexpressing the kinase-dead mutant D227A (D/A)-PI4P5-K Iα. Similar results were obtained with replicative and single-cycle HIV-1 particles. HIV-1 infection was also inhibited by knockdown of endogenous expression of PI4P5-K Iα. These data indicate that PI4P5-K Iα-mediated PIP_2 production is crucial for HIV-1 entry and the early steps of infection in permissive lymphocytes. ***The Journal of Immunology,*** **2008, 181: 6882–6888.**

Phosphatidylinositol (4, 5)-bisphosphate (PIP_2)[3] is a second messenger that binds, through its phosphorylated headgroup, to a variety of effector molecules and regulates their function and cellular localization. Several highly conserved phosphoinositide-binding sequences have been characterized, among which are the pleckstrin homology domain (PHD) and band four-point-one ezrin-radixin-moesin domain (1, 2). PIP_2 has also been reported to bind to a plethora of actin-binding proteins (3, 4), suggesting that it may control the link between the plasma membrane and cortical actin cytoskeleton (5, 6).

The major route for PIP_2 synthesis is the phosphorylation of phosphatidylinositol 4-phosphate (PI4P) by type I phosphatidylinositol 4-phosphate 5-kinases (PI4P5-K I) (7–9). Three PI4P5-K I isoforms (Iα, Iβ, and Iγ) have been reported, each having alternative splice variants (10, 11). This variety suggests that differential regulation of PI4P5-K I isoforms may enable cells to direct PIP_2 production for specific processes and to specific locations. Lymphocytes express two PI4P5-K I isoforms, PI4P5-K Iα and Iβ (7). Human PI4P5-K Iα has been shown to specifically localize to Rac1-induced membrane ruffles, whereas PI4P5-K Iβ is excluded from these structures and is detected primarily in cytosolic vesicular structures (12, 13). It therefore appears that PI4P5-K Iα is primarily involved in the production of PIP_2 related to the plasma membrane.

Plasma membrane-associated PIP_2 has been shown to be crucial for HIV-1 viral assembly (14–16). Anchorage of HIV-1 assembly to the plasma membrane is mediated by the PIP_2-dependent membrane localization of the Gag precursor protein $Pr55_{Gag}$ (14). Disruption of PIP_2 by overexpression of polyphosphoinositide 5-phosphatase IV (5ptaseIV) induces a redistribution of mature Gag from the plasma membrane to CD63-positive compartments and markedly reduces HIV-1 viral production (15, 17).

As the initial barrier to viral entry, the plasma membrane is also of fundamental importance in the initial stages of the viral cycle; however, little is known about the potential role of PIP_2 during viral attachment and entry to the target cell. In this study, we examine whether PI4P5-K Iα and associated PIP_2 production affect the early steps of the HIV-1 cycle. Our results suggest that HIV-1 viral particles, through the action of envelope (Env)-gp120 viral protein, trigger PIP_2 production in a PI4P5-K Iα-dependent manner during viral contact. Overexpression of wild-type (wt) PI4P5-K Iα enhances Env-induced PIP_2 production and HIV-1 infection, whereas these events are impaired by overexpression of an inactive mutant or PI4P5-K Iα silencing. These findings indicate that PI4P5-K Iα-mediated PIP_2 production is involved in the regulation of HIV-1 viral infection during the first steps of the viral cycle, before viral assembly and egress.

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[3] Abbreviations used in this paper: PIP_2, phosphatidylinositol 4,5-biphosphate; PI4P5-K type Iα, phosphatidylinositol 4-phosphate 5-kinase type Iα; PHD, pleckstrin homology domain; Env, envelope; wt, wild type; HA, hemagglutinin; MOI, multiplicity of infection; siRNA, short interference RNA.

Materials and Methods

Cells

The human $CD4^+$/$CXCR4^+$ CEM (acute lymphoblastic T cell leukemia) cell line was cultured in RPMI 1640 culture medium-10% FCS. The Jurkat cell line expressing X4-tropic HIV-1-Hxbc2 Env under tetracycline-off regulation, the fusion-inactive control Jurkat ΔKS cells expressing only the Rev viral protein, and CEM.NKR-CCR5 permissive cells were kindly provided by National Institutes of Health-AIDS Reagent Program. Human PBLs were isolated from healthy donor blood by Ficoll-Hypaque density gradient centrifugation (GE Healthcare). The PBLs were activated over 3 days with 1 μg/ml PHA (Murex Diagnostics) and then cultured with IL-2 (6 U/ml) as described (18).

Abs and reagents

The anti-CD4 HP2/6 mAb and non-neutralizing Ab were as described (18). Neutralizing anti-CD4 (OKT4A) was purchased from Ortho Diagnostic. The anti-CD3 OKT3 mAb was a gift from B. Alarcón (Centro de Biología Molecular, Madrid, Spain). The HRP-labeled anti-PIP_2 mAb Z-H045 was from Echelon Biosciences. The anti-PI4P5-K I (E-16, sc-11783), the anti-PI4P5-K Iα (C-17, sc-11774), and the anti-influenza hemagglutinin (HA) mAb (F-7, sc-7392) were from Santa Cruz Biotechnology. The cell tracker CMAC was from Molecular Probes. Recombinant soluble X4-tropic (rs)-HIV-1 gp120 viral protein (rs-gp120)-IIIB was obtained from either National Institutes of Health AIDS Research and Reference Reagent Program or Innogenetics, and was produced in either baculovirus or *Escherichia coli*, respectively. Both viral proteins similarly induced the production of PIP_2 at a dose of 5 μg/ml. AMD3100 was purchased from Sigma-Aldrich.

Type I PI4P5-K recombinant DNA constructs and cell transfection

Constructs encoding HA-tagged wt-PI4P5-K Iα, HA-tagged wt-PI4P5-K Iβ, and the HA-tagged kinase-dead mutants D227A (D/A)-PI4P5-K Iα and D268A-PI4P5-K Iβ were kindly provided by Dr. C. Lewis Cantley (Harvard Institute of Medicine, Boston, MA). The N-terminally GFP-tagged PHD from phosphatidylinositol-specific-phospholipase $C\gamma_1$ (GFP-PHD) was kindly provided by Dr. T. Balla (National Institutes of Health, Bethesda, MA). The PI4P5-K Iα and GFP-PHD constructs were nucleofected into cells using AMAXA kits (Amaxa), and cells were used 24 h posttransfection.

PIP_2 production

Cells (1×10^6) were equilibrated in RMPI 1640 complete medium containing 10 mM LiCl and then exposed to 5 μg/ml $rs\text{-}gp120_{IIIB}$. For the blocking experiments, cells were pretreated with neutralizing anti-CD4 or AMD3100 for 30 min at 37°C. Cells were lysed in MES buffer (10 mM MES (pH 7.4), 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM NaF, and a protease inhibitor mixture (Roche Diagnostics)) containing 1% SDS and 10 mM LiCl at different point times (from 0 to 2 h). Lysates were spotted onto nitrocellulose membranes with a dot-blot apparatus (Bio-Rad), and PIP_2 bands were probed with the anti-PIP_2 Ab Z-H045.

Luciferase viral entry assay

Luciferase-HIV-1 viral particles deficient for replication were kindly provided by Dr. Gummuluru (Boston University, Boston, MA). Replication-deficient viral particles were derived by the luciferase expressing reporter virus HIV/Δnef/Δenv/luc+ (which contains the *luciferase* gene inserted into the *nef* ORF and does not express *env* glycoprotein (19)) with a CXCR4-tropic (Lai) *env* glycoprotein. Virus stocks were generated by PolyFect transient transfection of 293T cells (20) Two days posttransfection, cell-free virus-containing supernatants were clarified of cell debris, concentrated by centrifugation ($16{,}000 \times g$, 1 h at 4°C), and stored at −80°C until required. HIV-1 virus preparations were titrated by ELISA and determination of the $p24_{Gag}$ content. Untreated or nucleofected CEM or PBLs (activated over 2 days with PHA (1 μg/ml)) were infected with a synchronous dose of luciferase-based virus for 2 h. Virus was removed by washing infected cells. After 32 h of infection, luciferase activity was determined with a luciferase assay kit (Promega) and a 1450 MicroBeta Luminiscence Counter (Walax, Trilux). Protein contents were measured by the bicinchoninic acid method (BCA protein assay kit; Pierce) according to the manufacturer's instructions.

HIV-1 preparation and infection

Preparation of $HIV\text{-}1_{NL4.3}$ and measurement of viral replication were performed as described (18) Highly infectious preparations of $HIV\text{-}1_{NL4.3}$ viral strain were generated by several consecutive passages of the original HIV-1 isolates in PBMC. Briefly, PBMCs were infected with one synchronous dose of $HIV\text{-}1_{NL4.3}$, and culture supernatants were recovered 3 days later and stored at −70°C. Freshly thawed aliquots were filtered through 0.22-μm filters before use. $HIV\text{-}1_{NL4.3}$ entry and multiplicity of infection (MOI, 1) was assayed in PHA (1 μg/ml)-activated PBLs or CEM T cells over 90 min. Cells were then trypsinized and extensively washed with fresh medium to remove viral input. Next, infected cells were kept in culture and viral entry and infection was monitored every 48 h by measuring the concentration of p24 in the culture supernatant by enzyme-linked immunosorbent assay (INNOTEST HIV-1 Ag mAb; Innogenetic). When indicated, permissive cells were pretreated with neutralizing anti-CD4 mAb (5 μg/ml) or 3′-azido-2′,3′-dideoxythymidine (5 μM) before addition of virus.

Production of nonreplicative viral particles containing BlaM-Vpr

X4- or R5-tropic HIV-1 viral particles deficient for replication and containing the BlaM-Vpr chimera were produced by cotransfecting 293T cells (70% confluence) in 75 cm^2 flasks the following vectors: pNL4–3.Luc.R-E- (20 μg; National Institutes of Health-AIDS Reagent Program); a CXCR4-tropic (HXB2-env; National Institutes of Health-AIDS Reagent Program) or CCR5-tropic (pCAGGS SF162 gp160; National Institutes of Health-AIDS Reagent Program) *env* glycoprotein vector(10 μg); and the pCMV-BlaM-Vpr vector (10 μg). The BlaM-Vpr chimera was kindly provided by Dr. Warner C. Greene (University of California, San Francisco, CA). Cotransduction of the pNL4–3.Luc.R-E- (20 μg) vector with the pHEF-VSV-G (10 μg; National Institutes of Health-AIDS Reagent Program) and pCMV-BlaM-Vpr (10 μg) vectors was used to generate nonreplicative viral particles that fuse with cells in a VSV-G-dependent manner. Viral plasmids were transduced in 293T cells by using linear polyethylenimine with an average molecular mass of 25kDa (PEI25k) (Polyscience). For this, viral plasmids were first dissolved in 1/10 of the final tissue culture volume of DMEM, free of serum and antibiotics. The PEI25k was prepared as a 1-mg/ml solution in water and adjusted to neutral pH. After addition of PEI25k to the viral plasmids (at a plasmid: PEI25k ratio of 1:5 (w/w)), the solution was mixed immediately, incubated for 20–30 min at room temperature, and then added to 293T cells in culture. After 4 h, the medium was changed to RPMI 1640, supplemented with 10% FCS and antibiotics, and the cells were cultivated to allow viral production. Viruses were harvested 40 h posttransfection. The supernatant was clarified by centrifugation at 3000 rpm for 30 min. Virions were then stored at −80°C. Viral stocks were normalized by p24-Gag content measured by ELISA (Innogenetics).

Virion-based fusion assay

A total of 1×10^6 CEM.NKR-CCR5 permissive cells were incubated 3 h with equivalent viral inputs of BlaM-Vpr-containing virions (500 ng of p24) in 500 μl RPMI 1640 medium. Cells were then extensively washed to remove free virions and incubated (1 h, room temperature) with CCF2-AM loading mix, as recommended by the manufacturer (GeneBLAzer detection kit; Invitrogen). Next, excess dye was washed off and cells were incubated for 16 h at room temperature before fixation with 4% paraformaldehyde. Then, 8×10^5 cells were placed in a 96-well plate per each experimental condition. The associated emission light to cleaved CCF2 (blue; 447 nm) and intact CCF2 probe (green; 520 nm) was measured. The entry of Blam-Vpr containing virions was measured as the ratio of the maximum fluorescence intensity between cleaved and intact CCF2. Thereby, an increase in this ratio indicates more fused viruses with target cells. The percentage of 100% of infection was determined by measuring the fluorescence intensities of intact and cleaved CCF2 probe in control infected cells (scrambled or pCDNA.3 transfected cells) and subtracting the background blue and green fluorescecence ratio determined in noninfected cells (without β-lactamase activity), as proposed by the manufacturer virus-infected (GeneBLAzer detection kit; Invitrogen).

Western blot

Treated cells were resuspended in 60 μl MES buffer, boiled for 5 min, and immunoblotted with anti-HA specific Ab. Protein bands were analyzed using the LAS-1000 CCD system and Image Gauge 3.4 software (Fuji Photo Film).

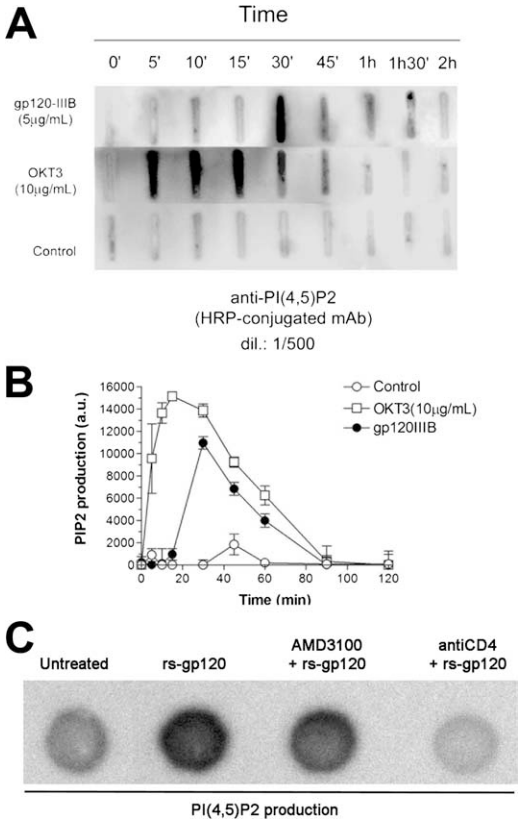

FIGURE 1. HIV-1-Env gp120 viral protein triggers PIP_2 production through CD4. *A*, Dot-blot analysis of rs-$gp120_{IIIB}$-induced PIP_2 production (*gp120-IIIB*), PIP_2 production after CD3 engagement (*OKT3*), and basal PIP_2 (untreated cells; *Control*) in CEM cells; a representative experiment of three is shown. CEM T cells were treated with 5 μg/ml rs-gp120 during the time-course. *B*, Quantification of PIP_2 production in three independent experiments. Data are means ± SEM. *C*, Dot-blot analysis of rs-$gp120_{IIIB}$-induced PIP_2 production in CEM T cells in the presence or absence of a neutralizing anti-CD4 mAb (OKT4A) or the CXCR4 antagonist AMD3100. A representative experiment of three is shown.

Immunofluorescence monitoring of PIP_2 during fusion-pore formation

For HIV-1 Env-mediated PIP_2 accumulation during fusion-pore formation, CMAC-charged Hxbc2-Env+ cells and nucleofected CEM cells were cocultured for 30 min (1:1 ratio) at 37°C. PIP_2-enriched plasma membranes during Env-mediated membrane fusion were monitored with the GFP-PHD biosensor (21). CMAC diffusion from Hxbc2-Env+ cells toward contacting cells was quantified by confocal microscopy.

mRNA silencing

Double-stranded short interference RNAs (siRNA) were generated against the following mRNA sequences of PI4P5-K Iα: 5′-uaggccaua gaaguguuga-3′ (*siRNA-1*; PI4P5-K Iα) and 5′-acacaguacucaguugaua-3′ (*siRNA-2*; PI4P5-K Iα) (Eurogentec). siRNA (1 μM) was nucleofected into cells, and assays were performed 24 h later. Irrelevant scrambled siRNA (Eurogentec) served as a control. Interference of PI4P-5K Iα expression was sustained for at least 96 h.

Results

HIV-1 Env-gp120 viral protein specifically triggers PIP_2 production in target cells

To assess the ability of HIV-1 to activate PIP_2 production in T cells, we incubated permissive $CD4^+$/$CXCR4^+$ CEM T cells with recombinant soluble X4-tropic (rs)-HIV-1 gp120 viral protein (rs-gp120). Cells were harvested over a time course (0–2 h), and cell lysates were analyzed for PIP_2 production (Fig. 1). Although untreated control cells did not accumulate PIP_2, treatment with rs-$gp120_{IIIB}$ specifically triggered PIP_2 production, with a peak at 30 min followed by a rapid decline (Fig. 1*A* and quantified in *B*). Correct metabolism of PIP_2 was confirmed in parallel positive control experiments with CD3; specific CD3 engagement with OKT3 Ab induced a strong peak of PIP_2 production after 10–15 min, as described (22), which thereafter declined (Fig. 1, *A* and *B*).

To further analyze gp120 induction of PIP_2 production, we performed the experiment in the presence of either a neutralizing anti-CD4 mAb or the CXCR4 antagonist AMD3100, to specifically block gp120 signal through CD4 or CXCR4. Although AMD3100 was functionally inhibiting CXCR4 signaling (data not shown), there was no significant effect in PIP_2 production after of rs-gp120 incubation. However, exposure to the neutralizing anti-CD4 mAb (OKT4A) effectively blocked gp120-induced PIP_2 production (Fig. 1*C*).

We next analyzed whether PIP_2 is produced during HIV-1 Env-mediated membrane fusion. To address this point, we used a cell-to-cell fusion system in which target T cells (CEM) are coincubated with Env+ cells (Hxbc2 Jurkat). To monitor PIP_2 localization during cell-to-cell contacts, target cells were transfected with GFP-PHD, which acts as a PIP_2 biosensor (21) (Fig. 2*A*). We also analyzed Env-induced membrane fusion by monitoring the diffusion of the cytoplasmic probe CMAC from charged-Hxbc2-Env+ cells to fused target cells. After 30 min of cell-to-cell contact, PIP_2 in the target cell was concentrated at the contact area with the Env+ cell, to where F-actin was also recruited (Fig. 2*A*). A similar redistribution to the cell membrane area in contact with Env+ cells was observed for endogenous PI4P5-K Iα (Fig. 2*C*). The specificity of these results was demonstrated by substituting the Hxbc2 cells with ΔKS cells, which lack a functional viral Env protein. Neither PIP_2 nor F-actin accumulated at heterotypic non-fused cell-cell contacts (Fig. 2*B*). These data suggest that HIV-1 Env specifically produces PIP_2 in permissive cells during the first contacts.

PI4P5-K Iα mediates HIV-1 Env-mediated PIP_2 production

In mammalian cells, PIP_2 is synthesized by type I PI4P5-K enzymes (7, 23). We therefore examined the involvement of PI4P5-K I in HIV-1-gp120-mediated PIP_2 production in permissive T cells. CEM cells were transfected with N-terminally HA-tagged versions of either the catalytically active wt PI4P5-K Iα, (wt) PI4P5-K Iβ, or the dominant-negative kinase-dead mutants D227A (D/A)-PI4P5-K Iα and D268A (D/A)-PI4P5-K Iβ (Fig. 3*A* and data not shown). Overexpression of wt-PI4P5-K Iα increased the amounts of PIP_2 produced upon exposure to rs-$gp120_{IIIB}$, 30 min (Fig. 3*B*). In contrast, overexpression of D/A-PI4P5-K Iα strongly impaired rs-$gp120_{IIIB}$-mediated PIP_2 production (Fig. 3*B*). Neither construct affected the production of PIP_2 by untreated cells (Fig. 3*B*). Although overexpression of wt-PI4P5-K Iβ also promoted HIV-1 Env-mediated PIP_2 production, its dead-mutant D/A-PI4P5-K Iβ had no effect on phosphoinositide production (Fig. 3*C*). The membrane expression of CD4 and CXCR4/CCR5 was unaffected by transfection with the PI4P5-K Iα constructs (Fig. 3*D*). These data therefore indicate the specific involvement of PI4P5-K Iα in HIV-1 Env-induced PIP_2 production.

HIV-1 infection of permissive lymphocytes requires the presence of functional PI4P5-K Iα

We next assessed the functional involvement of PI4P5-K Iα in HIV-1 infection. Primary lymphocytes overexpressing the active PI4P5-K Iα construct showed enhanced HIV-1 viral production at

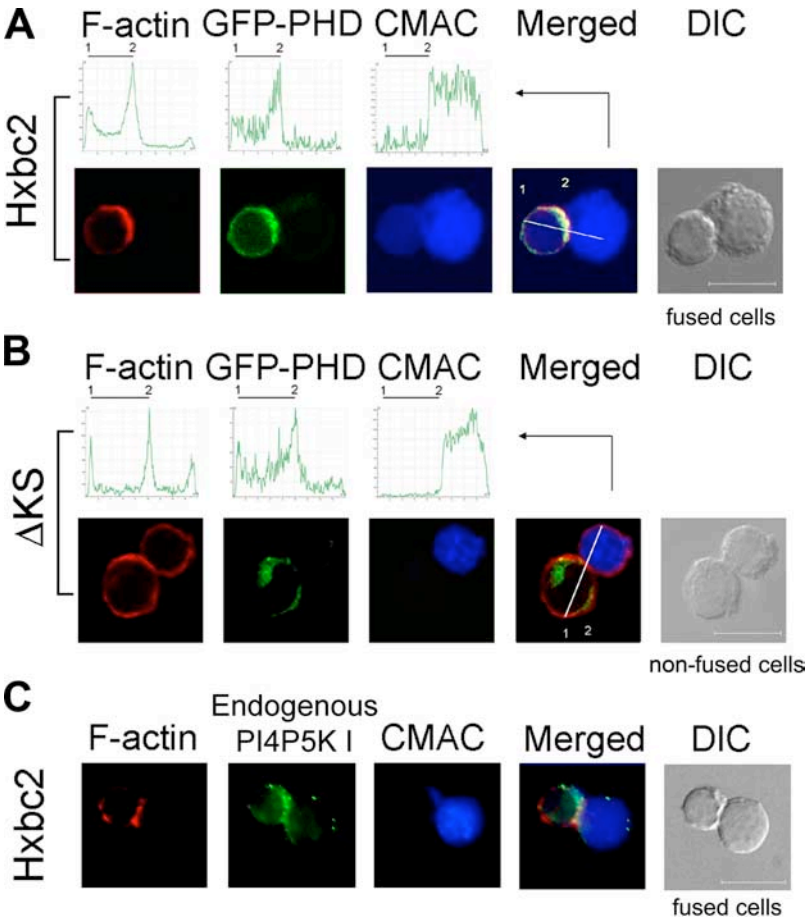

FIGURE 2. PIP_2 is produced in an Env-dependent manner during HIV-1 Env-mediated membrane fusion. *A*, Confocal microscopy and quantification of Hxbc2-Env-mediated PIP_2 production during membrane fusion after 30-min coculture. Accumulation of PIP_2 was determined from the fluorescence of GFP-PHD biosonde. Images show localization of F-actin and GFP-PHD at cell-cell contacts and diffusion of the cytoplasmic probe CMAC from Jurkat-derived Hxbc2 cells (blue cell to *right*) to the target CEM T cell (*left*). CMAC diffusion from Env+ Hxbc2 cells to target cells and F-actin and GFP-PHD accumulation were quantified along lines (see *Merged* pictures) drawn through the region of cell-cell contact. Points 1 and 2 indicate target-cell measurement points and cell diameters. *B*, Confocal microscopy showing the same analysis for heterotypic nonfused cell-cell contacts between CEM T cells and ΔKS (Env-) cells after 30-min coculture. Images show the absence of F-actin and GFP-PHD localization at cell-cell contacts and of CMAC diffusion from Jurkat-derived ΔKS cells (blue cell to *right*) to the target T cell (*left*). *xy* mid-sections are shown. Bar, 10 μm. *C*, Immunofluorescence microscopy images of F-actin and PI4P5-K Iα localization at cell-cell contacts and CMAC diffusion from Jurkat-derived Hxbc2 cells (blue *right-hand cell*) to the target CEM T cell (*left cell*) are shown.

3 days postinfection, whereas infection was inhibited in cells overexpressing the D/A-PI4P5-K Iα construct (Fig. 4*A*). These observations could be due to the action of PIP_2 during viral assembly and budding, so to explore the role of PI4P5-K Iα in the early steps of viral entry we conducted experiments with a single-cycle virus. Nonreplicative HIV-1 viral particles bearing the *Luc* reporter gene were incubated with CEM permissive cells (Fig. 4*B*) or primary T cells (Fig. 4*C*) overexpressing wt- or D/A-PI4P5-K Iα. Remarkably, over-expression of active PI4P5-K Iα clearly enhanced HIV-1 viral entry compared with control GFP-transfected cells in

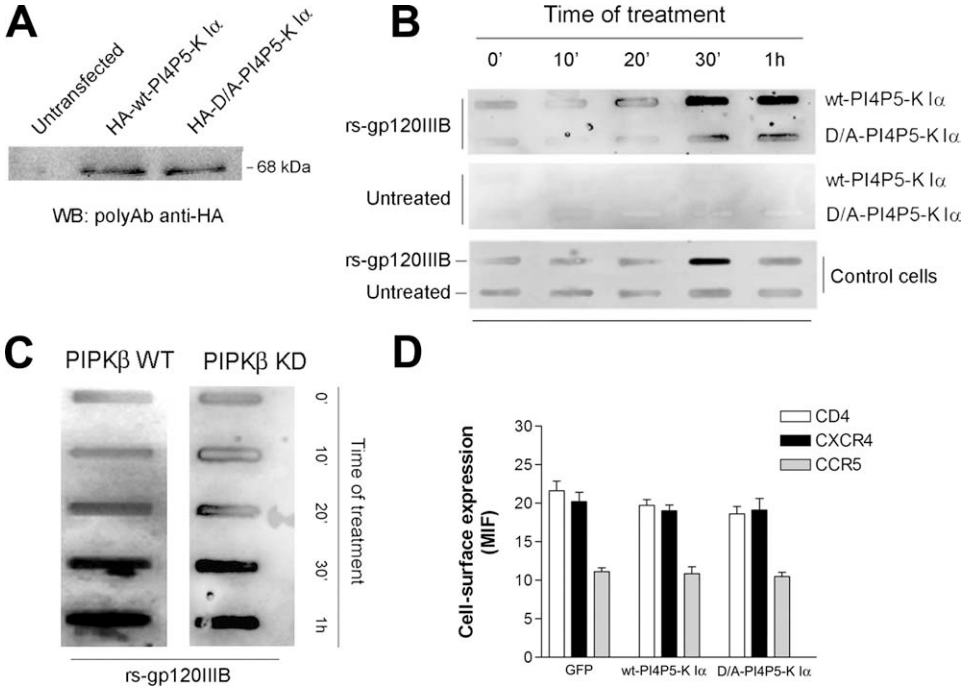

FIGURE 3. PI4P5-kinase Iα mediates specific HIV-1-Env-induced PIP_2 production. *A*, Western blot analysis of overexpressed HA-tagged wt-PI4P5-K Iα and dominant negative kinase-inactive D/A-PI4P5-K Iα in CEM cells. *B*, Dot-blot analysis of rs-$gp120_{IIIB}$-mediated PIP_2 production in CEM T cells nucleofected with wt- or D/A-PI4P5-K Iα, and compared with untransfected (control) cells. Cells were treated with 5 μg/ml rs-gp120 during the time course. Basal PIP_2 is shown for nucleofected and non-nucleofected cells (*Untreated*). A representative experiment of three is shown. *C*, Dot-blot analysis of rs-$gp120_{IIIB}$-mediated PIP_2 production in cells nucleofected with wt-PI4P5K Iβ or D/A-PI4P5-K Iβ (KD). *D*, Flow cytometry analysis of CD4, CXCR4, and CCR5 cell surface expression in PI4P5-K Iα overexpressing cells. GFP = nucleofection control.

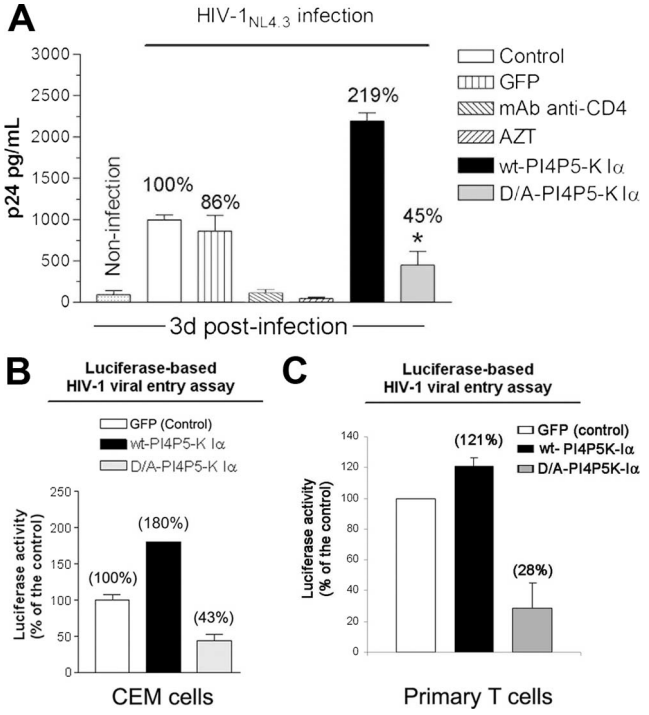

FIGURE 4. PI4P5-K Iα is required for viral entry and infection of HIV-1 particles. *A*, HIV-1 infection (MOI, 1) in primary T cells overexpressing wt-PI4P5-K Iα or D/A-PI4P5-K Iα (∗ indicates $p < 0.05$). The infection levels for non-nucleofected (Control) and GFP-nucleofected (GFP) cells are shown, and where indicated infection was inhibited with anti-CD4 mAb or 3′-azido-2′,3′-dideoxythymidine. Data are the means ± SEM of three independent experiments conducted in triplicate. *B*, Luciferase-based HIV-1 viral entry assay in CEM T cells overexpressing wt- or D/A-PI4P5-K Iα. Data are means ± SEM of three independent experiments. *C*, Luciferase-based HIV-1 viral entry assay in primary T cell blasts overexpressing wt- or D/A-PI4P5-K Iα, compared with cells transfected with GFP protein (Control). Data are means ± SEM of three independent experiments, conducted in triplicate.

CEM and primary T cells (Fig. 4, *B* and *C*). In contrast, overexpression of the dead-mutant D/A-PI4P5-K Iα strongly inhibited viral entry (Fig. 4, *B* and *C*).

PI4P5-K Iα regulates HIV-1 viral entry into target cells

To further analyze the role of PI4P5-K Iα during HIV-1 infection, we have performed viral fusion and entry experiments by using R5- and X4-tropic HIV-1 viral particles containing the BlaM-Vpr chimera (24). These chimera virions have been designed to specifically study the first steps of viral infection, since β-lactamase activity directly correlates with viral entry. Control, wt-, or dm-PI4P5-K Iα-transfected CEM.NKR-CCR5 cells were incubated (3 h) with equivalent viral inputs of X4-tropic or R5-tropic virions containing BlaM-Vpr fusion protein (Fig. 5*A*). Concurring with previous data, cells overexpressing wt- PI4P5-K Iα were more susceptible to viral entry, whereas in cells overexpressing D/A-PI4P5-K Iα viral entry was strongly reduced. These data indicate that functional PI4P5-K Iα is required for efficient HIV-1 viral fusion and entry, independently of the viral tropism.

As a control of the specificity of PI4P5-K Iα regulation of HIV-1-induced membrane fusion, we repeated the experiment this time using BlaM-Vpr virions pseudotyped with VSV-G Env, which allows viruses to enter the target cell using the endocytic lower pH pathway. As expected, VSV-G viral entry was independent of PI4P5-K Iα kinase activity (Fig. 5*B*).

PI4P5-K Iα knockdown impairs HIV-1 viral infection and entry

To confirm the involvement of PI4P5-K Iα in HIV-1-mediated viral infection, we suppressed expression of the endogenous enzyme by overexpressing a specific siRNA. Two different siRNA oligonucleotides were tested and yielded knockdown of 30–70% (Fig. 6*A*). The basal cell surface expression levels of the CD4 and CXCR4 cell receptors for HIV-1 infection were not affected in PI4P5-K Iα-silenced cells (Fig. 6*B*). Using the siRNA-2 oligonucleotide, we observed that the lack of endogenous PI4P5-K Iα significantly inhibited HIV-1 infection in CEM cells (Fig. 6*C*).

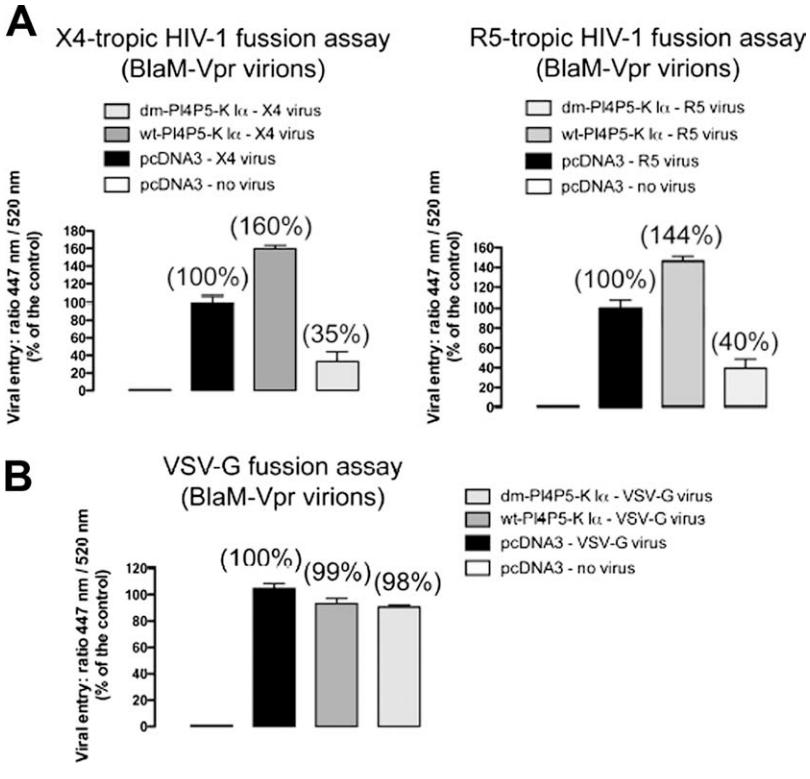

FIGURE 5. PI4P5-K Iα regulates HIV-1 viral entry into target cells. *A*, PI4P5-K Iα specifically affects the early steps of viral infection. Control, wt-, or dm-PI4P5-K Iα-transfected CEM. NKR-CCR5 cells were incubated for 3 h with equivalent viral inputs (determined by standard p24-ELISA) of X4-tropic (*left panel*) or R5-tropic (*right panel*) pNL4–3.Luc.R-E- virions containing BlaM-Vpr fusion protein. After adsorption for 3 h, a fraction of cells were treated with CCF2-AM and analyzed by fluorescence spectrophotometry after 16 h. *B*, VSV-G-mediated viral fusion is independent of PI4P5-K Iα. VSV-G virions containing the BlaM-Vpr fusion protein were used to control for the specificity of wt- or dm-PI4P5-K Iα-mediated effects on HIV-1 viral fusion. The percentages of HIV-1-fused cells were determined by measuring the ratio of blue (447 nm; cleaved CCF2) to green (520 nm; intact CCF2) fluorescent signals in target cells. Each assay was done in triplicate and results are representative of three independent experiments.

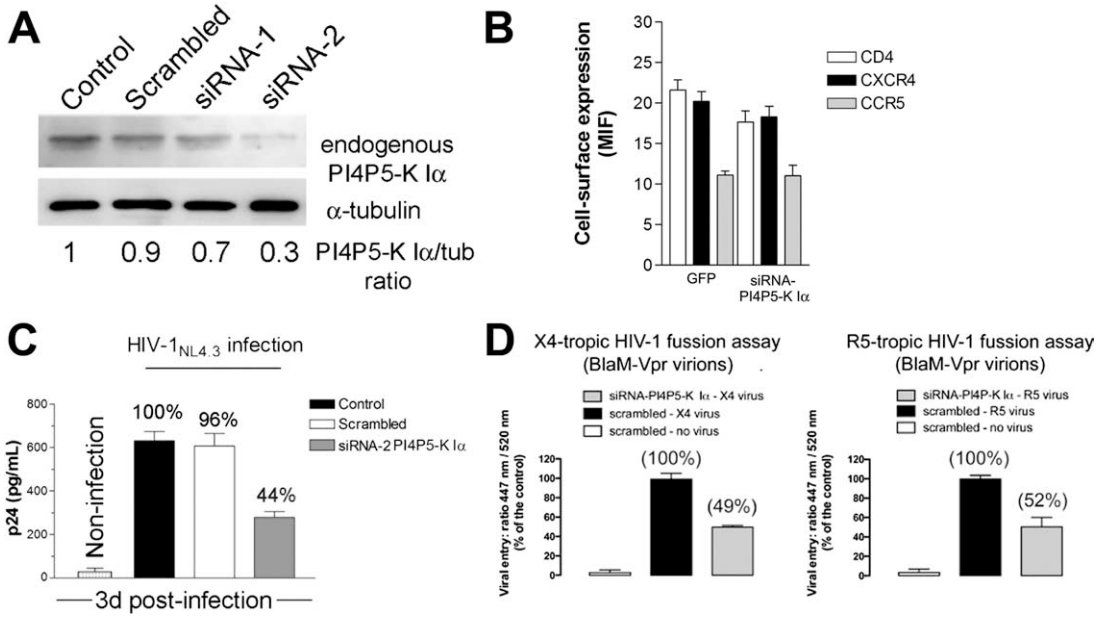

FIGURE 6. PI4P5-kinase Iα regulates HIV-1 infection at the step of viral entry, confirmed by specific mRNA silencing. *A*, Western blot analysis of specific silencing of endogenous PI4P5-K Iα (oligonucleotides *siRNA-1* and *siRNA-2*) 24 h after siRNA-oligonucleotide nucleofection of CEM cells. Silencing is quantified as the band-intensity ratio of PI4P5-K Iα to α-tubulin. A representative experiment of three is shown. *B*, Flow cytometry analysis of CD4, CXCR4, and CCR5 cell surface expression in PI4P5-K Iα-silenced cells. GFP = nucleofection control. Data are means ± SEM of three independent experiments, conducted in triplicate. *C*, HIV-1 viral infection (MOI, 1) in CEM cells in which PI4P5-K α is silenced with siRNA-2. Control, untransfected cells; scrambled, nucleofection with an unrelated scrambled siRNA oligonucleotide. Data are means ± SEM of two independent experiments, conducted in triplicate. *D*, Specific PI4P5-K Iα knock-down decreases entry into target cells of X4- or R5-tropic BlaM-Vpr-virion proteins. CEM.NKR-CCR5 cells were incubated for 3 h with equivalent viral inputs, as determined by standard p24-ELISA. After adsorption for 3 h, a fraction of cells were treated with CCF2-AM and analyzed by fluorescence spectrophotometry after 16 h. The percentages of HIV-1-fused cells were determined by measuring the ratio of blue (447 nm; cleaved CCF2) to green (520 nm; intact CCF2) fluorescent signals in target cells. Each assay was done in triplicate and results are representative of three independent experiments.

These data suggest that functional PI4P5-K Iα is required for efficient viral infection. Using the BlaM-Vpr tool to infect CEM T cells silenced for PI4P5-K Iα, we confirmed that PI4P5-K Iα is required for HIV-1-specific entry (Fig. 6*D*). This effect is independent of HIV-1 Env tropism, which suggests that PI4P5-K Iα regulates a process in target cells that is generally required during the entry stage of HIV-1 infection.

Discussion

The results presented in this study provide the first demonstration that efficient early HIV-1 infection requires T lymphocyte expression of PI4P5-K Iα and associated synthesis of PIP_2. Overexpression of functional wt-PI4P5-K Iα favors an increased production of PIP_2 in response to HIV-1 Env that is concomitant with an increase in viral entry, as observed with single-cycle HIV-1 particles (both luciferase and β-lactamase reporter systems). Conversely, overexpression of the kinase-dead mutant D/A-PI4P5-K Iα or knockdown of endogenous PI4P5-K Iα impairs viral-induced PIP_2 production and HIV-1 entry. An important role has previously been established for PIP_2 metabolism in HIV-1 assembly and egress through the localization of the Gag precursor protein $Pr55_{Gag}$ to the plasma membrane (15, 16). The current results show that PI4P5-K Iα regulates viral entry, therefore indicating that PIP_2 regulates HIV-1 infection throughout the viral cycle.

Three different isoforms of PI4P5-K I have been cloned and described; both α and β isoforms have similar molecular masses (68 kDa) (7). Nevertheless, little is known about differences in their function and regulation. The regulatory mechanism operating in specific processes could be due to different subcellular localizations of these isoforms. It has been described that human PI4P5-K Iα was found preferentially in membrane ruffles, whereas PI4P5-K Iβ isoform was localized in vesicular structures in the cellular cytoplasm (12), where it is implicated in regulation of endocytic vesicles formation. This could represent a coordinate mechanism to regulate PIP_2 production by the cell.

PI4P5-K I has been implicated in regulation of actin dynamics through the interaction of PIP_2 with cytoskeletal molecules such as gelsolin, profilin, filamin A, and ERM proteins (4, 7, 25). The PIP_2 pool at the inner leaflet of the plasma membrane is thought to promote the recruitment of these actin-related proteins to specific membrane domains or to induce activating or inhibitory conformational changes within target proteins. PI4P5K I has been shown to be involved in several processes related to this cortical actin redistribution, including cell polarity (25) cytokinesis (26), phagocytosis (27), and cell migration and the formation of membrane protrusions (28). In this regard, HIV-1 viral entry occurs at specific cell surface areas enriched in actin and viral receptors, such as ruffles and microvilli (29, 30). The behavior of these structures is governed by cortical actin dynamics, which in turn depend on the activity of actin cytoskeleton associated proteins. It is therefore possible that PI4P5-K I might regulate the formation of these structures and promote HIV-1 entry.

PIP_2 has important regulatory roles in many cellular processes (31, 32) and affects protein organization and acts as a precursor for second messengers such as DAG, IP3, and PIP3 (33). It is thought that specificity of PIP_2 action may in part be achieved through spatial confinement in pools in the plasma membrane (34, 35), where it could affect plasma membrane fluidity and protein organization (36). HIV-1 Env-mediated PIP_2 production during viral

entry might therefore alter plasma membrane fluidity or viral receptor organization to favor initial HIV-1/cell interactions and promote subsequent fusion.

PIP_2 has also been found to protect against cell apoptosis, alone or in a complex with gelsolin, by directly inhibiting the initiator caspases 8 and 9, as well as by binding and inhibiting their common effector, caspase 3 (37, 38). Therefore it is plausible that HIV-1-triggered synthesis of PIP_2 during the first steps of the viral cycle may serve to ensure cell viability and, therefore, efficient viral infection and propagation. Moreover, it is conceivable that the efficiency and/or kinetics of CD4/gp120-mediated PIP_2 production may affect the fusion process established between HIV-1 viral particles and target cells. In this way, cell signals that regulate viral activation of PI4P5-K Iα and subsequent PIP_2 production could be crucial for the control of HIV-1 viral entry and infection.

In sum, we propose that signals that regulate PI4P5-K Iα-dependent PIP_2 production and related cellular processes may control HIV-1 viral infection by regulating the first steps of viral cycle and may represent new targets for the blockade of HIV-1 infection.

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Disclosures

The authors have no financial conflict of interest.

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