The Phosphatidylinositol 3-Kinase p110α/PTEN Signaling Pathway Is Crucial for HIV-1 Entry

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Human immunodeficiency virus type 1 (HIV-1) drives multiple signaling pathways to facilitate its cellular entry and replication. The interaction between HIV-1 envelope (env) protein and target cell surface CD4 first activates the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, and the subsequent interaction between HIV-1 env glycoprotein and CCR5/CXCR4 coreceptors establishes viral fusion and entry. Four isoforms of the class-I PI3K catalytic subunits (p110α, p110β, p110γ, and p110δ) have been identified so far, but the isoform(s) involved in the HIV-1 entry is still unknown. This study aimed to identify the PI3K isoform(s) using recently developed isoform-specific inhibitors and the roles of their negative regulators, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and homology 2 domain-containing inositol-5-phosphatase 1 (SHIP1), in HIV-1 infection. We found that the PI3K p110α isoform-specific inhibitor P1K-75 suppressed HIV-1 entry in HIV-1-permissive T cells, PM1 cells, and TZM-bl cells (HeLa cell-derived indicator cells that coexpress CD4, CCR5, and CXCR4) and decreased the HIV-1-induced phosphorylation of Akt. Moreover, wild-type PTEN (but neither phosphatase-deficient PTEN nor wild-type SHIP1) was a key regulator of HIV-1 entry. Cell-to-cell fusion by HIV-1 env–CD4 interaction was suppressed in the presence of P110K p110α-specific inhibitor. These data suggest that the PI3K p110α/PTEN signaling pathway is indispensable for HIV-1 entry, including HIV-1 env-mediated cell-to-cell fusion.

Key words human immunodeficiency virus infection; phosphatidylinositol 3-kinase; phosphatase

INTRODUCTION

The entry of human immunodeficiency virus type 1 (HIV-1) is dependent on the expression of its principle receptor CD4 and coreceptors, CCR5 and CXCR4.1–3 HIV-1 can activate multiple signaling pathways within a target cell to facilitate viral entry and replication. Interaction of HIV-1 viral envelope (env) glycoprotein-CD4 stimulates CD4 receptor phosphorylation on its serine residues,4 which leads to receptor endocytosis by its dissociation from noncovalently interacting tyrosine kinase p56 lck.5,6 Subsequent interaction between envelope glycoprotein 120 (gp120) and the coreceptor is thought to lead to fusion between the viral and target cell membranes, namely viral entry.7 The G protein-coupled receptor (GPCR)-type chemokine receptor could also activate several different intracellular effectors through pertussis toxin-sensitive Giα, including low-molecular-weight guanosine 5'-triphosphatases (GTPases) (Ras and Rho), phosphatidylinositol 3-kinases (PI3Ks), and mitogen-activated protein kinase (MAPK).8 Because these pathways ultimately regulate functions such as cytoskeletal rearrangement, cell survival, differentiation, and activation of transcription, HIV-1 gp120-induced signal transduction may facilitate virus infection.

PI3Ks catalyze the production of phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P3) from phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2). Growth factors and hormones trigger this phosphorylation, which in turn coordinates cell growth, cell cycle entry, cell migration, and cell survival.9–11 Class I PI3Ks are heterodimeric proteins, consisting of a 110 kDa catalytic subunit (p110) and an adaptor/regulatory subunit, and have been subgrouped into class IA and class IB PI3Ks. Class IA PI3Ks, consisting of catalytic subunits (p110α, p110β, p110δ), are involved in receptor tyrosine kinase pathways, whereas class IB PI3K (p110γ) acts downstream of GPCRs,12,13 A major effector of PI3K signaling is the serine/threonine kinase Akt (also known as protein kinase B). Glycogen synthase kinase 3α/β (GSK-3α/β), which is a downstream target of Akt, is also a serine/threonine protein kinase that mediates cellular responses to growth factors such as cell cycle transition, cell growth, and cell migration.

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN; also called MMAC1 or TEP1) is a cloned tumor suppressor gene,14 which preferentially dephosphorylates lipid substrates.15,16 Dephosphorylation of the PI3K product, PtdIns(3,4,5)P3, by PTEN on the 3’ of the inositol ring leads to decreased levels of these phospholipids and a simultaneous reduction in Akt activity.15,17 Loss of PTEN expression has been observed in multiple tumor types.18–20 On the other hand, homology 2 domain-containing inositol-5-phosphatase (SHIP) is a recently identified lipid phosphatase, which is mainly expressed in hematopoietic cells in response to cytokine and growth factor stimulation.19 Dephosphorylation of the PI3K product PtdIns(3,4,5)P3, by SHIP on the 5’ of the inositol ring leads to decreased levels of these phospholipids and a simultaneous reduction in Akt and Bruton’s tyrosine kinase activity,20 leading to the inhibition of hematopoietic cell proliferation and survival.21 Recent studies have demonstrated that gp120–CD4 interaction in vitro results in rapid activation of the p85 adaptor subunit of PI3K, and both chemokine receptors and the Src
protein tyrosine kinase lck are required for full PI3K activity.\textsuperscript{5,23} These data indicate that PI3K signaling is significant for HIV-1 infection. Moreover, chemokine receptors are GPCRs, and the activation of PI3K by gp120 binding is impaired by pertussis toxin pretreatment.\textsuperscript{24} Therefore, gp120 binding to CD4 and its coreceptors may activate class IB PI3K (p110\textsubscript{γ}) rather than class IA PI3Ks (p110\textsubscript{α}, p110\textsubscript{β}, p110\textsubscript{δ}). However, it is still unclear which PI3K isoform plays a critical role in HIV-1 entry and HIV-1 env-mediated cell-to-cell fusion. Using a panel of novel isoform-selective PI3K inhibitors, we found that the p110\textsubscript{α} isoform-specific inhibitor PIK-75 suppressed HIV-1 entry, and PTEN (not SHIP1) played a critical role in HIV-1 infection. These results reveal critical roles of the PI3K p110\textsubscript{α}/PTEN signaling pathway for HIV-1 infection.

MATERIALS AND METHODS

Reagents and Plasmids Pan-PI3K p110 inhibitor LY294002 and p110\textsubscript{α} inhibitor PIK-75 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The p110\textsubscript{β} inhibitor TGX-221 and p110\textsubscript{γ} inhibitor AS605240 were obtained from Chemedea (Ridgewood, NJ, U.S.A.). The p110\textsubscript{δ} inhibitor IC87114 was purchased from BioVision (Mountain View, CA, U.S.A.). PTEN-coding sequences, pMX-PTEN-WT and pMX-PTEN-C124S mutant (phosphatase-deficient mutant), were introduced into a retroviral pMX-puro vector. TZM-bl cells were infected with the supernatants from 293T cells that had been transfected with these pMX expression plasmids. Cells were examined 48h post-infection for HIV-1 infection experiments. Wild-type SHIP1 (SHIP1-WT) and a phosphatase-inactive mutant of SHIP (SHIP1-D675A)\textsuperscript{25} cDNA were cloned into a pcDNA3.1 hygro(+)-N-FLAG vector. The HIV-1 proviral expression vector pJR-FL and the HIV-1 env expression vector pJR-FL env were kindly provided by Dr. Yoshio Koyanagi (Kyoto University).

Cell Culture and Transfection Reactions A TZM-bl cell line was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Health (Rockville, MD, U.S.A.). The two CD4-expressing T cell lines, CEM and PM1, were maintained in RPMI1640 media (Sigma-Aldrich, U.S.A.) supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/mL penicillin, and 100 \( \mu \)g/mL streptomycin. TZM-bl and 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% (v/v) FCS and antibiotics. All cells were maintained at 37°C with 5% CO\textsubscript{2}. Transfection of cells on a 35-mm dish (2.0 \times 10\textsuperscript{4} cells) was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.).

Cell Viability Test 3-(4,5-Dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) assays were applied for testing cell viability. First, 1 \times 10\textsuperscript{5} cells (per well) were seeded into a 96-well dish and incubated with or without various concentrations of PI3K inhibitors for 48h at 37°C in 5% CO\textsubscript{2}. Subsequently, 10 \( \mu \)L MTT (0.5 mg/mL final concentration) was added to each well. After 4h, 100 \( \mu \)L of 0.01 N HCl in isopropanol was added to dissolve the crystals. Absorbance at 570 nm was measured.

HIV-1 Preparation and Infection To prepare HIV-1 viruses, 293T cells were transfected with a HIV-1 cDNA-carrying plasmid pJR-FL (a gift from Dr. Yoshio Koyanagi, Kyoto University) using a Lipofectamine 2000. The viral supernatants were harvested 48h later and stored at –70°C. Stock virus titers were determined using an HIV-1 p24 antigen enzyme-linked immunosorbent assay kit (ZeptoMetrix Corp., Buffalo, NY, U.S.A.). We facilitated HIV-1 infection using a standard spin infection technique for PM1 cells (1010 g for 1h at 18°C) or by incubating TZM-bl cells with the virus at 37°C for 2h.\textsuperscript{26}

Immunoblotting Immunoblotting analyses were carried out using a standard protocol and primary antibodies against phospho-Akt [S473], total Akt, p110\textsubscript{α}, p110\textsubscript{β}, p110\textsubscript{δ}, phospho-GSK-3\textbeta [Ser9], total GSK-3\textbeta (Cell Signaling Technology, Beverly, MA, U.S.A.), PTEN (Cascade Bioscience, Winchester, MA, U.S.A.), p110\textsubscript{γ} [H-219] (Santa Cruz), and β-actin (Sigma). Primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (Cell Signaling) with standard chemiluminescent procedures.

Detection of Intracellular p24 by Flow Cytometric Analysis HIV-1-infected cells were fixed with 4% paraformaldehyde for 10min, permeabilized with 0.1% saponin (Sigma) for 10min, and stained with fluorescein isothiocyanate (FITC)-conjugated anti-HIV-1 p24 monoclonal antibody (mAb) (Beckman Coulter, Fullerton, CA, U.S.A.) for 30min on ice. After washing, the cells were analyzed using an LSRII flow cytometer (BD Bioscience, San Jose, CA, U.S.A.). Phorbol 12-myristate 13-acetate (PMA)-activated ACH-2 cells (lymphoid HIV-1 persistently infected cells) were used as positive control for HIV-1 infection/intracellular HIV-1 p24 protein expression. Data analysis were performed with FlowJo software (Tree Star, San Carlos, CA, U.S.A.).

Determination of Virus Infectivity TZM-bl cell line is derived from a HeLa cell clone that was engineered to express CD4, CCR5 and CXCR4\textsuperscript{27} and to contain integrated reporter gene for firefly luciferase under the control of the HIV-1 long terminal repeat promoter.\textsuperscript{28} For determination of HIV-1 infectivity, TZM-bl cells were incubated with a pan-PI3K inhibitor (LY294002) or PI3K p110 isoform-specific inhibitors (PIK-75 for p110\textsubscript{α}, TGX-221 for p110\textsubscript{β}) for 24h at 37°C, and then infected with HIV-1 JR-FL supernatants for 48h at 37°C. Thereafter, the cells were washed once with PBS, lysed in 200 \( \mu \)L Reporter Lysis Buffer (Promega, Madison, WI, U.S.A.) on ice. After washing, the cells were analyzed using an LSRII flow cytometer (BD Bioscience, San Jose, CA, U.S.A.).

HIV-1 Env-Mediated Cell-to-Cell Fusion Assay The 293T cells transfected with pJR-FL env were labeled with a green fluorescent dye, PKK67 (2 \( \mu \)M). PM1 or TZM-bl cells were labeled with a red fluorescent dye, PKK126 (2 \( \mu \)M). The two cell lines were mixed, and after 12h, cell-to-cell fusion was detected by fluorescence microscopy using a BZ-8100 fluorescence microscope (KEYENCE, Osaka, Japan) and flow cytometric analysis using an LSRII flow cytometer.
Cytotoxicity of these cells. 7) To evaluate the impact of PI3K isoform on HIV-1 env-mediated cell-to-cell fusion, an assay system was established using PM1 cells and HIV-1 env protein-expressing 293T cells. When the effector (293T-expressing env) and the target (PM1) cells were labeled with green (PKH67) and red (PKH26) fluorescent dyes, respectively, then cell-to-cell fusion can be detected as double-positive (yellow) labeling (Fig. 5). As seen in Fig. 6a, green signals were observed around the plasma membranes of 293T env cells and red signals in PM1 target cells whereas, after co-culture, numerous large synctia (white arrowheads) were observed as yellow signals (Fig. 6a). When FACS was employed for quantitative analyses (Fig. 6b), both LY294002 and PIK-75 (but not TGX-221, AS605240, IC87114) markedly suppressed the numbers of double-positive cells (cell-to-cell fusion with target and effector cells) (Fig. 6c). The similar results were obtained when TZM-bl cells were employed as target cells (Figs. 7a–c). These data support the finding that PI3K p110α has a critical role in HIV-1 env-mediated cell-to-cell fusion.

**DISCUSSION**

PI3K is known to play an important role in the inhibition of apoptosis, down-regulation of MHC, and latency for HIV-1 infection. In addition, Akt and GSK-3β, the downstream effectors of PI3K, have been shown to play critical roles in HIV-1 infectivity. However, the PI3K isoforms involved in this process have not been clarified and moreover, little is known about the roles of phosphatase PTEN or SHIP1 in PI3K signaling.

In conclusion, we demonstrated that the PI3K isoforms involved in HIV-1 infection are p110α and p110β and that the PTEN-C124S construct is capable of suppressing HIV-1 entry into the TZM-bl cells. We also showed that the PI3K pathway plays an important role in HIV-1 entry into these cells. Furthermore, we demonstrated that the PTEN signaling pathway plays an important role in HIV-1 entry into these cells.
Among four isoforms, p110α and p110β share a high degree of sequence homology and are widely expressed among tissues, but growing evidence suggests differences in their physiological roles. On the other hand, p110γ and p110δ are predominantly found in the hematopoietic system. The differential physiological roles of PI3K isoforms were shown by targeted disruptions of p110α, p110β, p110γ, and p110δ. To date, several studies including those using subtype selective inhibitors have accumulated detailed information on their functional differences; however, the isoform(s) involved in the HIV-1 entry step remained unknown until the current study.

**Fig. 2.** PI3K p110α Is Required for HIV-1 Infection in PM1 Cells

(a) Expression levels of PI3K p110 isoforms in CEM, PM1, and TZM-bl cells. Whole-cell lysates were analyzed by Western blotting to detect expression of p110α, p110β, p110γ, and p110δ (a loading control). (b) Cell viability (MTT) tests in PM1 cells to evaluate PI3K p110 inhibitors. Different doses of pan (LY294002) and isoform-specific inhibitors (PIK-75 for p110α, TGX-221 for p110β, AS605240 for p110γ, IC87114 for p110δ) were applied to cells for 24 h. All treatments were performed in triplicates and error bars indicate standard deviation, n = 3. *p < 0.05 in Student’s t-test. (c) PM1 cells were incubated with the indicated concentrations of pan and isoform-specific PI3K p110 inhibitors for 24 h prior to infection with HIV-1 JR-FL supernatants. After 48 h, the cells were stained with p24-FITC and analyzed for the expression of intracellular HIV-1 gag protein p24 by flow cytometry. PMA-activated ACH-2 cells were used as positive control for HIV-1 infection. Population percentages for p24-positive cells are presented. (d) PM1 cells were incubated with LY294002 (10 µM) and other inhibitors (30 nM) for 24 h at 37°C prior to infection with HIV-1 JR-FL supernatants. After 2 h, whole-cell lysates were extracted and analyzed by Western blotting.
rent study. We utilized four isoform-selective PI3K inhibitors that have previously been extensively characterized: PIK-75 (p110α-specific),35,37 TGX-221 (p110β-specific),38 IC87114 (p110δ-specific)39 and AS605240 (p110γ-specific).40 We found that only LY294002 (pan inhibitor) and PIK-75 inhibited HIV entry (Figs. 2c, 3b), Akt phosphorylation (Figs. 2d, 3c), and cell-to-cell fusion induced by HIV-1 env protein (Figs. 6c, 7c).

Although some studies suggest that gp120 binding to CD4 and its coreceptors stimulates the activation of PI3K p110γ rather than PI3K p110α, p110β and p110δ,41 a p110γ inhibitor (AS605240) did not inhibit HIV-1 entry and HIV-1 env-mediated cell-to-cell fusion (Figs. 6, 7). Moreover, a recent report mentioned that the PI3K agonist specific to p110α but not isoforms p110β, δ, or γ reactivated latent HIV-1 in several cell-line models of latency.42 In fact, p110γ inhibition by AS605240 did not abrogate PI3K/Akt signaling as measured by p-Akt (Ser473) levels in PM1 cell (Fig. 2d). Further studies (e.g., with higher AS605240 concentrations) are needed to clarify the role of p110γ. Taken together, these data suggest that PI3K p110α, not p110γ, may be necessary for HIV-1 entry steps, especially cell-to-cell fusion.

Our study also demonstrated that the damping of PI3K signaling by PTEN but not SHIP1 interferes with HIV-1 entry through the Akt/GSK-3β-dependent pathway (Fig. 4).
Thus, HIV-1 entry could also be driven by the regulation of PTEN expression. Several possibilities may explain the failure of SHIP1 to block the PI3K/Akt pathway (Figs. 4c, d). First, PtdIns(3,4,5)P$_3$ by PTEN rather than SHIP1 might render the products fully inactive for Akt binding and activation. Thirdly, PTEN may possess much higher phosphatase activity (potency) than SHIP1 due to its higher affinity toward PtdIns(3,4,5)P$_3$. It was shown previously that transfection of PTEN-WT induces the dephosphorylation of Akt and GSK-3β more potently, compared with SHIP1-WT. It should be noted that SHIP1 could not suppress the PI3K/Akt pathway in myeloma cells.

The PI3k/Akt signaling in host cells could bidirectionally modulate viral infection. During Epstein–Barr virus (EBV) infection, EBV protein (LMP2A) induces CpG island methylation of the PTEN promoter through signal transducers and activator of transcription 3 (STAT3) phosphorylation, thereby upregulating PI3k/Akt signaling to confer host cell survival. In addition, hepatitis B virus X protein downregulates PTEN expression and activates Akt signaling during liver cell infection. Meanwhile, during infection with vesicular stomatitis virus, the viral M protein could inactivate Akt and thereby blunt the interferon-dependent antiviral responses in host cells. In our study, PI3K/Akt pathway as well as PTEN expression suppressed HIV-1 entry and HIV-1 env-mediated cell-to-cell fusion. Recently, genome-wild (RNA interference (RNAi) and short hairpin RNA) screening for host defense factors required for HIV-1 replication identified Akt signaling. Taken together, the PI3K p110α/Akt/GSK-3β/PTEN signaling pathway is crucial for HIV-1 entry as well as host cell defense against HIV-1. Therapeutic (either pharmacological or genetic) approaches to treat HIV patients through PI3K p110α/Akt/GSK-3β/PTEN pathways await further investigation.
Fig. 6. HIV-1 Env-Mediated Cell-to-Cell Fusion Induced HIV-1 Envelope Protein Is Necessary for PI3K p110α Signaling in PM1 Cells
(a and b) Monitoring of double-positive signals after co-culture of 293T cells expressing HIV-1 JR-FL envelope (PKH67: green) and PM1 cells (PKH26: red). PM1 cells were labeled with PKH26 and then treated with pan (LY294002) and isoform-specific inhibitors (PIK-75 and TGX-221) for 2 h at 37°C. After washing, labeled PM1 cells were co-cultured with PKH67-labeled env-expressing 293T cells. After 12 h, images were obtained using a fluorescence microscope (a) and FACS analysis (b). A scale bar indicates 50 µm and arrowheads indicate double-positive (yellow) cells in (a). Population percentages for PKH26 and PKH67 double-positive cells are presented in (b). (c) Effects of various PI3K p110 inhibitors on the double-positive cell populations. Data are the means ± standard deviations of three independent experiments performed in triplicate. ***p < 0.001; Student’s t-test. (Color figure can be accessed in the online version.)

Fig. 7. HIV-1 Envelope Protein-Mediated Cell-to-Cell Fusion Is Necessary for PI3K p110α Signaling in TZM-bl Cells
(a and b) Monitoring of the double-positive signal after co-culture of 293T cells expressing HIV-1 envelope (PKH67: green) and TZM-bl cells (PKH26: red). TZM-bl cells were labeled with PKH26 and then treated with pan (LY294002) and isoform-specific inhibitors, PIK-75 and TGX-221, for 2 h at 37°C. After washing, labeled TZM-bl cells were co-cultured with PKH67-labeled env-expressing 293T cells. After 12 h, images were obtained using a fluorescence microscope (a) and FACS analysis (b). A scale bar indicates 50 µm and arrowheads indicate double-positive (yellow) cells in (a). Population percentages for PKH26 and PKH67 double-positive cells are presented in (b). (c) Effects of various PI3K p110 inhibitors on the double-positive cell populations. Data are the means ± standard deviations of three independent experiments performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001; Student’s t-test. (Color figure can be accessed in the online version.)
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Conflict of Interest  The authors declare no conflict of interest.

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