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The Phosphatidylinositol 3-Kinase p110 α /PTEN Signaling Pathway Is Crucial for HIV-1 EntryKoichi Hamada,^{*,a,b} Yosuke Maeda,^c Akihiro Mizutani,^a and Seiji Okada^b

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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 (p110 α , p110 β , p110 γ , and p110 δ) 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 p110 α isoform-specific inhibitor PIK-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 PI3K p110 α -specific inhibitor. These data suggest that the PI3K p110 α /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 G α i, 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 110kDa 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 (p110 α , p110 β , p110 δ), are involved in receptor tyrosine kinase pathways, whereas class IB PI3K (p110 γ) 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 3 α/β (GSK-3 α/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.^{14,18)} 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,^{20,21)} leading to the inhibition of hematopoietic cell proliferation and survival.²²⁾

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

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protein tyrosine kinase lck are required for full PI3K activity.^{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.²⁴⁾ Therefore, gp120 binding to CD4 and its coreceptors may activate class IB PI3K (p110 γ) rather than class IA PI3Ks (p110 α , p110 β , p110 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 α isoform-specific inhibitor PIK-75 suppressed HIV-1 entry, and PTEN (not SHP1) played a critical role in HIV-1 infection. These results reveal critical roles of the PI3K p110 α /PTEN signaling pathway for HIV-1 infection.

MATERIALS AND METHODS

Reagents and Plasmids Pan-PI3K p110 inhibitor LY294002 and p110 α inhibitor PIK-75 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The p110 β inhibitor TGX-221 and p110 γ inhibitor AS605240 were obtained from Chemdea (Ridgewood, NJ, U.S.A.). The p110 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 48 h post-infection for HIV-1 infection experiments. Wild-type SHP1 (SHP1-WT) and a phosphatase-inactive mutant of SHP1 (SHP1-D675A)²⁵⁾ cDNA were subcloned 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 μ 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% CO₂. Transfection of cells on a 35-mm dish (2.0 $\times$ 10⁵ cells) was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) in accordance with the manufacturer's protocol.

Cell Viability Test 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were applied for testing cell viability. First, 1 $\times$ 10⁴ cells (per well) were seeded into a 96-well dish and incubated with or without various concentrations of PI3K inhibitors for 48 h at 37°C in 5% CO₂. Subsequently, 10 μ L MTT (0.5 mg/mL final concentration) was added to each well. After 4 h, 100 μ 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 48 h 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 1 h at 18°C) or by incubating TZM-bl cells with the virus at 37°C for 2 h.²⁶⁾

Immunoblotting Immunoblotting analyses were carried out using a standard protocol and primary antibodies against phospho-Akt [S473], total Akt, p110 α , p110 β , p110 δ , phospho-GSK-3 β [Ser9], total GSK-3 β (Cell Signaling Technology, Beverly, MA, U.S.A.), PTEN (Cascade Bioscience, Winchester, MA, U.S.A.), p110 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 10 min, permeabilized with 0.1% saponin (Sigma) for 10 min, and stained with fluorescein isothiocyanate (FITC)-conjugated anti-HIV-1 p24 monoclonal antibody (mAb) (Beckman Coulter, Fullerton, CA, U.S.A.) for 30 min on ice. After washing, the cells were analyzed using a 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 were analyzed 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 CXCR4²⁷⁾ and to contain integrated reporter gene for firefly luciferase under the control of the HIV-1 long terminal repeat promoter.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 α , TGX-221 for p110 β) for 24 h at 37°C, and then infected with HIV-1 JR-FL supernatants for 48 h at 37°C. Thereafter, the cells were washed once with PBS, lysed in 200 μ L Reporter Lysis Buffer (Promega, Madison, WI, U.S.A.) and frozen at -70°C until use. Samples were freeze/thawed 2 times and a half portion of the lysis solution was analyzed using a luminometer.²⁸⁾

HIV-1 Env-Mediated Cell-to-Cell Fusion Assay The 293T cells transfected with pJR-FL env were labeled with a green fluorescent dye, PKH67 (2 μ M). PM1 or TZM-bl cells were labeled with a red fluorescent dye, PKH26 (2 μ M). The two cell lines were mixed, and after 12 h, cell-to-cell fusion was detected by fluorescence microscopy using a BZ-8100 fluorescence microscope (KEYENCE, Osaka, Japan) and flow cytometric analysis using a LSRII flow cytometer.

RESULTS

HIV-1 Activates the Akt/GSK-3 β Pathway To investigate the specific function of PI3K during HIV-1 infection, PI3K downstream targets, Akt and GSK-3 β , were analyzed by immunoblotting. In order to detect HIV-1 entry, we utilized the indicator cell line TZM-bl (also called JC57BL-13), a HeLa cell derivative that expresses high levels of CD4/CXCR4/

Fig. 1. HIV-1 Infection Activates the PI3K/Akt/GSK-3 β Signaling Pathway

(a) TZM-bl cells were infected with HIV-1 JR-FL supernatants at 37°C for the indicated time periods. Total protein was extracted and analyzed by Western blotting using anti-pAkt, anti-Akt, anti-pGSK-3 β , anti-GSK-3 β , and anti- β -actin antibodies. (b) PM1 cells were infected with HIV-1 JR-FL supernatants using a spin method for 1 h at 18°C and incubated at 37°C for the indicated time. Data are the representative of three independent experiments and estimated molecular weight sizes are shown.

CCR5, and stably carries an LTR-driven firefly luciferase.^{27,28)} HIV-1 infection induced the phosphorylation of Akt and GSK-3 β within 30 min, which was sustained for 180 min (Fig. 1a). Similar results were obtained in HIV-1 permissive T cells, PM1 cells (Fig. 1b). These results demonstrated that HIV-1 infection activates the two PI3K downstream effectors, Akt and GSK-3 β .

PI3K p110 α Plays an Important Role in HIV-1 Infection First we investigated PI3K isoform expression. Western blotting analyses revealed that all PI3K isoforms p110 α , p110 β , p110 γ , and p110 δ were co-expressed in CEM and PM1 cells, whereas only p110 α , p110 β , and p110 δ (to a lesser extent) were expressed in TZM-bl cells (Fig. 2a). Next, we investigated the effects of various PI3K p110 inhibitors, a pan-inhibitor as well as isoform-specific inhibitors. The reported IC₅₀ values of each PI3K p110 inhibitor against each PI10 isoform are summarized in Table 1.²⁹⁾ The pan-PI3K inhibitor LY294002 suppressed PM1 cell viability in a dose-dependent manner (Fig. 2b), as reported previously,³⁰⁾ whereas other PI3K isoform-specific inhibitors (p110 α -specific PIK-75, p110 β -specific TGX-221, p110 γ -specific IC87114, and p110 δ -specific AS605240) did not suppress cell viability at the given concentrations (Fig. 2b). In contrast, LY294002 suppressed HIV-1 entry as reported previously³⁰⁾ but PIK-75 (not TGX-221, AS605240, IC87114) suppressed HIV-1 entry more potently (Fig. 2c). Accordingly, only LY294002 and PIK-75 inhibited Akt activation induced by HIV-1 infection (Fig. 2d).

We further investigated the effects of LY294002, PIK-75, and TGX-221 on TZM-bl cells that mainly express p110 α and p110 β . TZM-bl cells were resistant to the applied concentrations of these three reagents in cell viability tests (Fig. 3a). When a highly sensitive luciferase reporter gene assay was employed to detect HIV-1 entry, only LY294002 and PIK-75 potently inhibited virus entry (Fig. 3b). Accordingly, only LY294002 and PIK-75 inhibited Akt activation in infected TZM-bl cells (Fig. 3c). Taken together, the PI3K p110 α /Akt signaling pathway plays an important role in HIV-1 entry into these cells.

3-Phosphatase PTEN but Not 5-Phosphatase SHIP1 Regulates HIV-1 Infection PTEN negatively regulates the PI3K pathway by dephosphorylating its product PtdIns(3,4,5)P₃ at

the D3 position, leading to the suppression of cell growth and other PI3K/Akt-dependent processes.¹⁵⁾ To investigate the role of PTEN in HIV-1 entry, TZM-bl cells were transfected with a wild-type PTEN cDNA construct (PTEN-WT) or a lipid and protein phosphatase-deficient PTEN cDNA construct (PTEN-C124S),³¹⁾ and 2 d later, the cells were treated with HIV-1 JR-FL supernatants for 48 h. Luciferase activities (namely HIV-1 entry levels) were much lower in PTEN-WT-transfected cells than those in PTEN-C124S-transfected cells (Fig. 4a). In accordance, phosphorylation levels of both Akt and GSK-3 β were much lower in PTEN-WT-transfected cells than those in PTEN-C124S-transfected cells (Fig. 4b).

SHIP1 is known to act as 5-phosphatase against PtdIns(3,4,5)P₃, thereby counteracting the PI3K pathway. We thus examined the impact of SHIP1 on HIV-1 entry into TZM-bl cells.²⁵⁾ The transfection of either SHIP1-WT or phosphatase-inactive SHIP1-D675A did not affect HIV entry (Fig. 4c) or Akt/GSK-3 β activation (Fig. 4d). Taken together, the PI3K p110 α /Akt/PTEN signaling pathway is important for HIV-1 entry into these cells.

PI3K p110 α Signaling Is Necessary for HIV-1 Env-Mediated Cell-to-Cell Fusion The interaction between HIV-1 envelope gp120 and the coreceptor on a target cell is considered to be important for subsequent membrane fusion between the virus and the target cell.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 syncytia (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 p110 α 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.^{8,24,32)} In addition, Akt and GSK-3 β , the downstream effectors of PI3K, have been shown to play critical roles in HIV-1 infectivity.³³⁾ 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 HIV-1 entry. In this study, functional differences between specific PI3K p110 isoforms in HIV-1 entry were examined. The study demonstrated that: 1) PI3K p110 α signaling is critical in HIV-1 entry, 2) PTEN but not SHIP1 negatively regulates HIV-1 entry, and 3) PI3K p110 α signaling regulates cell-to-cell fusion induced by HIV-1 env protein. To the best of our knowledge, this is the first report demonstrating the critical roles PI3K “p110 α ”/PTEN signaling in HIV-1 entry and HIV-1

Fig. 2. PI3K p110 α 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 p110 α , p110 β , p110 γ , p110 δ , and β -actin (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 p110 α , TGX-221 for p110 β , AS605240 for p110 γ , IC87114 for p110 δ) 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 μ M) 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.

env-mediated cell-to-cell fusion.

Among four isoforms, p110 α and p110 β share a high degree of sequence homology and are widely expressed among tissues, but growing evidence suggests differences in their physiological roles.¹⁰ On the other hand, p110 γ and p110 δ are predominantly found in the hematopoietic system. The dif-

ferential physiological roles of PI3K isoforms were shown by targeted disruptions of p110 α , p110 β , p110 γ and p110 δ .³⁴ To date, several studies including those using subtype selective inhibitors have accumulated detailed information on their functional differences^{35,36}; however, the isoform(s) involved in the HIV-1 entry step remained unknown until the cur-

Table 1. PI3K p110 Inhibitors (Pan and Isoform-Specific) and Their Reported IC₅₀ Values^{35,36)}

Inhibitors	Targets	IC ₅₀ (nM)			
		p110 α	p110 β	p110 γ	p110 δ
LY294002	Pan	730	310	6600	1060
PIK-75	p110 α	6	1300	76	510
TGX-221	p110 β	5000	5	3500	100
AS605240	p110 γ	60	270	8	300
IC87114	p110 δ	200000	16000	61000	130

Fig. 3. PI3K p110 α Is Required for HIV-1 Infection in T2M-bl Cells

(a) Cell viability test in PI3K p110 inhibitors in T2M-bl cells. MTT assays were performed in the presence of different doses of pan (LY294002) and isoform-specific inhibitors, PIK-75 and TGX-221, for 24 h. All treatments were performed in triplicates. Error bars indicate standard deviation, $n = 3$. (b) T2M-bl cells were incubated with pan (LY294002) and isoform-specific inhibitors, PIK-75 and TGX-221, for 24 h prior to infection with HIV-1 JR-FL supernatants. Luciferase activity in the cell lysates was measured. Data are the representative of three independent experiments. $**p < 0.01$, $***p < 0.001$; Student's t -test. (c) T2M-bl cells were incubated with pan (LY294002, 10 μ M) and isoform-specific inhibitors, PIK-75 and TGX-221 (30 nM each), for 24 h 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 (p110 α -specific),^{35,37)} TGX-221 (p110 β -specific),³⁸⁾ IC87114 (p110 δ -specific)³⁹⁾ and AS605240 (p110 γ -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 p110 γ rather than PI3K p110 α , p110 β and p110 δ ,⁴¹⁾ a p110 γ 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 p110 α but not isoforms p110 β , δ , or γ reactivated latent HIV-1 in several cell-line models of latency.⁴²⁾ In fact, p110 γ 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 p110 γ . Taken together, these data suggest that PI3K p110 α , not p110 γ , 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-3 β -dependent pathway (Fig. 4).

Fig. 4. PTEN Expression Interferes with HIV-1 Infection

(a) HIV-1 luciferase reporter assay in PTEN-expressing TZM-bl cells. PTEN expression sequences, pMX-PTEN wild-type (PTEN-WT) and pMX-PTEN phosphatase-deficient mutant (PTEN-C124S) were introduced into a retroviral pMX-puro vector. TZM-bl cells were infected with the supernatants of 293T cells transfected with pMX-PTEN-WT or pMX-PTEN-C124S. After 48h, TZM-bl cells were infected with HIV-1 JR-FL supernatants for 48h. Luciferase activities in the cell lysates were measured using a luminometer. Data are representative of three independent experiments. ** $p < 0.01$; Student's t -test. (b) Effects of PTEN-WT or PTEN-C124S expression on Akt/GSK-3 β activities in TZM-bl cells. After 48h, whole cells were lysed and cell extracts were analyzed by Western blotting. Data are the representative of three independent experiments. (c) HIV-1 luciferase reporter assay in SHIP1-expressing TZM-bl cells. TZM-bl cells were transfected with wild-type SHIP1 (SHIP1-WT) and phosphatase-inactive SHIP1-D675A mutant. After 48h, TZM-bl cells were infected with HIV-1 JR-FL supernatants for 48h. Luciferase activities in the lysates were measured. Data are the representative of three independent experiments. (d) Effects of SHIP1-WT or SHIP1-D675A expression on Akt/GSK-3 β activities in TZM-bl cells. Data are the representative of three independent experiments.

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)P₂, the product of SHIP, may play some compensatory role in maintaining Akt activation. Secondly, dephosphorylation of PtdIns(3,4,5)P₃ 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

Fig. 5. Scheme of the HIV-1 Env-Mediated Cell-to-Cell Fusion Assay

affinity toward PtdIns(3,4,5)P₃.⁴³⁾ It was shown previously that transfection of PTEN-WT induces the dephosphorylation of Akt and GSK-3 β more potently, compared with SHIP1-WT.²⁵⁾ It should be noted that SHIP1 could not suppress the PI3K/Akt pathway in myeloma cells.²⁵⁾

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.⁴⁴⁾ In addition, hepatitis B virus X protein downregulates PTEN expression and activates Akt signaling during liver cell infection.⁴⁵⁾ 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.⁴⁶⁾ In our study, PI3K p110 α inhibition as well as PTEN expression suppressed HIV-1 entry and HIV-1 env-mediated cell-to-cell fusion. Recently, genome-wide (RNA interference (RNAi) and short hairpin RNA) screening for host defense factors required for HIV-1 replication identified Akt signaling.^{47,48)} Taken together, the PI3K p110 α /Akt/GSK-3 β /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 p110 α /Akt/GSK-3 β /PTEN pathways await further investigation.

Fig. 6. HIV-1 Env-Mediated Cell-to-Cell Fusion Induced HIV-1 Envelope Protein Is Necessary for PI3K p110 α 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 μ m 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 $\pm$ standard deviations of three independent experiments performed in triplicates. *** p < 0.001 in 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 p110 α Signaling in T2M-bl Cells

(a and b) Monitoring of the double-positive signal after co-culture of 293T cells expressing HIV-1 envelope (PKH67: green) and T2M-bl cells (PKH26: red). T2M-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 T2M-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 μ m 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 $\pm$ 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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