

Supplemental Materials and Methods

Reagents

The anti-Tat antibodies used for immunofluorescence were either monoclonals from ABI (#13-161-100; discontinued) or santa cruz biotechnology (sc-65912) or polyclonal anti-Tat HXB2 (86 residues) antibodies that were raised in rabbits. The monoclonals used for ELISA were from abcam (#ab42359) or from the Center for AIDS reagents, NIBSC, England (#ARP352). Goat anti-p24 antibody was from Serotec, anti-GFP from abcam (#ab290), anti-Ki67 from Dako, anti-hsp90 from BD biosciences (#610418), anti-Histone H1 and anti-CD4 from santacruz (sc-8030 and sc-7219, respectively). Secondary antibodies were from Jackson ImmunoResearch. WGA conjugated to Cy5 was from Invitrogen. Tat peptides were from the Center for AIDS reagents (NIBSC, England). Chemicals were from Sigma-Aldrich and natural phospholipids from Avanti Polar Lipids. Ins(1,4,5)P₃, synthetic phosphoinositides and GST-PH_{PLC δ} used in ELISA and Biacore experiments was purchased from Echelon Biosciences Inc. GST-PH_{Akt} was from Upstate Biochemicals (# 27932). GST-PH_{PLC δ} used in ITC experiments was purified exactly as described (Watt et al., 2002). 10-DN was kindly provided by Erwin London (Stony Brook University, New York).

Construction of HIV-1 Tat variants

The EcoRI-BamHI fragment of the X4-tropic pNL4-3 molecular clone (AIDS reagents, contributor M. Martin, NCBI accession number AF324493) was subcloned and mutations were introduced by PCR using appropriate primers. First we restored a full 101 residues Tat protein by reverting the TAG stop codon at 8413 to TCG (Jeang et al., 1999). The Tat-W11Y mutation was obtained by changing the TGG to TAT at 5860. The entire subclone was sequenced to verify the absence of additional mutations, and was then inserted back in the infectious molecular clone. The same procedure was followed with the R5-tropic pNL (AD8; from AIDS reagents, contributor E.O. Freed, NCBI accession number AF004394). HIV-1 virions were produced by transfecting 293T cells. The transfection supernatant was then used to infect Jurkat CD4-CCR5 cells to obtain higher viral titers. The viral concentration was monitored by Gag p24 ELISA assay (Innotest, Ingen, France).

Cells, transfection and infection by HIV-1

The Jurkat clone E6-1 from the ATCC was transfected using electroporation as described (Vendeville et al., 2004). To prepare primary CD4⁺ T-cells, human blood from healthy volunteers was obtained from the local blood bank (EFS, Montpellier) and peripheral blood mononuclear cells were isolated using Ficoll Hypaque gradients (Bessoles et al., 2008). They were depleted of monocytes by adherence to plastic and CD4⁺ T-cells were recovered by positive isolation using the protocol provided by the kit manufacturer (Dyna-Invitrogen). They were then activated using phytohemagglutinin (1 µg/ml) for 24 h then interleukin-2 (50 U/ml) for 5-6 days, replacing the medium every 2-3 days before transfection using the Amaxa kit for activated T-cells, or infection by HIV-1. Infection of primary T-cells was performed with HIV-1 NL4-3 at a final m.o.i. of 0.1, equivalent to 3 µg of Gag p24. Viral production was quantified using p24 ELISA. Jurkat CD4-CCR5 cells (Centre for AIDS reagents, EVA programme, contributor Q. Sattentau) were infected with either AD8 (WT or 11Y mutant) or pNL4.3 (WT or 11Y mutant) overnight at the m.o.i. of 0.1. Cells were washed the next day and infection was monitored for 14 days using p24 ELISA.

Flow cytometry analysis

To assess coreceptor expression, $5 \cdot 10^5$ Jurkat CD4-CCR5 cells were resuspended in 100 µl of PBS containing 1% fetal calf serum (PBS/FCS) and anti-CXCR4 (MAB173, R&D systems), anti-CCR5 (2D7, BD Biosciences) or anti-dihydrofolate reductase (BD Biosciences) as control. After 40 min on ice, cells were washed with PBS/FCS and labelled with fluorescein-labeled goat anti-mouse antibodies as described for the primary antibody. Cells were finally washed with PBS and fixed with 1% paraformaldehyde in PBS before analysis on a FACScalibur machine using CellQuest software. Intracellular p24 staining was performed at room temperature. Cells were fixed for 20 min using 1% paraformaldehyde in phosphate-buffered saline (PBS), washed in PBS/FCS and incubated for 10 min in permeabilization buffer (PBS supplemented with 5 mM EDTA, 1% FCS and 0.2% saponin). Cells were then labelled with fluorescein-labelled anti-p24 antibody (KC57-FITC, Beckman Coulter) for 30 min in permeabilization buffer, washed and fixed using 1% paraformaldehyde in PBS before FACS analysis. At least 10000 events were collected for each sample.

Cell fractionation

Primary CD4⁺-T cells (10⁷) were transfected with a Tat vector alone (4 µg) or together with an effector plasmid (2 µg each). After 24 h, cells were collected, washed and fractionated using a subcellular protein fractionation kit as described by the manufacturer (Thermo Fisher) with the following modifications. Incubations for the preparation of cytosol and membrane fractions were performed on ice for 5 min, and a single nuclear fraction was prepared. Proteins were then precipitated (Wessel and Flugge, 1984), before separation by SDS-PAGE using tricine gels (Schagger, 2006). Proteins were electroblotted to a nitrocellulose membrane and detected using antibodies against Tat (sc-65912), hsp90 (BD-610418), histone H1 (sc-8030), CD4 (sc-7219) or EGFP (abcam290). Primary antibodies were labeled with secondary antibodies conjugated to horseradish peroxidase that was detected using ECL⁺ (GE Healthcare). Films were exposed within their linear range of detection and band quantification was performed using ImageQuant (GE Healthcare).

PI(4,5)P₂ binding assays

To prepare multilamellar vesicles (Barret et al., 2000), egg phosphatidylcholine (PC) was mixed with the indicated phosphoinositide in water before lyophilization overnight, resuspension at 20 mg/ml in 0.5 mM EGTA, 20 mM Hepes, pH 7.4 and 3 h incubation at 42°C. Tat (100 µM in degassed 0.5 mM EGTA, 15 mM β-mercaptoethanol, 20 mM Hepes, pH 7.4) was centrifuged before use. Tat (10 µM) was incubated with liposomes (0.5 mg/ml lipids) in 100 mM KCl, 20 mM Hepes, pH 7.4, for 1 h at room temperature before ultracentrifugation (100 000 x g, 10 min). Proteins from both supernatant and pellet were separated by SDS/PAGE. Gels were stained for band quantification using ImageQuant (GE Healthcare).

The binding of purified Tat or GST-PH_{PLCδ} to ELISA plates coated with different phosphoinositides was assayed as recommended by the manufacturer (Echelon Biosciences Inc.).

For SPR experiments, small unilamellar vesicles were prepared by sonication (Méré et al., 2005) from 75% PC, 20% phosphatidylethanolamine (PE) and 5% of the indicated phosphoinositide. Similar data were obtained when using phosphatidylglycerol (PG) instead of PE. The vesicle size was monitored between sonication periods using a Coulter Counter N4S apparatus until the diameter was 50-100 nm. The surface of a L1 sensor chip fitted on a Biacore 3000 biosensor was cleaned by 5 min injection of 20 mM CHAPS followed by 1 min

of 30% ethanol at a flow rate of 10 $\mu\text{l}/\text{min}$. Vesicles (20 μl at 3 mM lipid concentration in PBS) were then injected at 2 $\mu\text{l}/\text{min}$. The lipid layer was then washed at 100 $\mu\text{l}/\text{min}$ with 20 μl of 10 mM NaOH. The control for non-specific binding was performed using BSA (100 $\mu\text{g}/\text{mL}$ in PBS). Unless otherwise indicated, the experiments were performed using 200 nM Tat in HBS buffer (400 mM NaCl, 10 mM HEPES, pH 7.4). Affinities were estimated with a 1:1 langmuir fitting model using BIAevaluation software 4.1. Competition assays were performed by two injections of peptide (50 μM , 3 min) followed by an injection of 200 nM Tat. HCl 25 mM was used for the regeneration step.

For ITC analysis, Tat (0.1-0.5 μM in 150 mM NaCl, 50 mM potassium citrate, pH 7.0, termed citrate buffer) was placed in the 1.4 ml sample cell of a VP-ITC MicroCalorimeter from MicroCal Incorporated. Liposomes (PC/PG/PI(4,5)P₂ or PC/PG/PI(3,4,5)P₃ 75/20/5), diC8-PI(4,5)P₂ or Ins(1,4,5)P₃ were then injected at concentrations of 10 μM , 3 μM and 3 μM , respectively. We also titrated GST-PH_{PLC δ} (5.6 μM) by PI(4,5)P₂- liposomes or Ins(1,4,5)P₃ (110 or 75 μM in the syringe, respectively) and GST-PH_{Akt} (1 μM) by PI(3,4,5)P₂- liposomes (50 μM in the syringe). For each titration, 10 – 15 μl injections were used, at 5 min intervals, at 25°C. Injections were also performed in ITC buffer alone, and the resulting dilution heat was subtracted from the binding curves. Data were fit with Origin software and stoichiometry for protein binding to PIP_x liposomes was based on 100% lipid accessible in the liposome. For monolayer measurements, Tat insertion into the phospholipid monolayer was measured at 23 °C by monitoring the change in surface pressure (π) at constant surface area using a 10-ml circular Teflon trough and a small diameter wire probe fitted on a Kibron microtrough S instrument (Stahelin et al., 2003). A lipid monolayer containing PC/PG (75/25) or PC/PG/PI(4,5)P₂ (75/20/5) was spread onto the subphase composed of citrate buffer, until the desired initial surface pressure (π_0) was reached. After signal stabilization (~10 min), Tat (26 nM) was injected into the subphase and the increase in surface pressure ($\Delta\pi$) was monitored for 30 min while stirring the subphase at 60 rpm. Typically, the $\Delta\pi$ value reached a maximum after 20 min. The resulting $\Delta\pi$ was plotted versus π_0 , from which the critical surface pressure (π_c) was determined as the x-intercept (Stahelin et al., 2003).

For fluorescence measurements, a stock of lipid vesicles (4 mM lipid concentration) composed of PC/PS (75/25) or PC/PS/PI(4,5)P₂ (75/20/5), containing or not 10 mol % of 10-DN (Caputo and London, 2003) was prepared by sonication. Fluorescence was measured at room temperature on a PerkinElmer SS35 spectrofluorometer using semi-micro quartz cuvettes. Trp fluorescence intensity was measured at 331 nm using excitation at 280 nm.

Slit widths were set to 5 nm. For measurements, liposomes were diluted to 900 μM in citrate buffer and background fluorescence was recorded before adding Tat (2.5 μM) and recording the signal every 2 sec for 300 sec. Under such conditions, the Tat/available-PI(4,5) P_2 ratio is ~ 10 . Background intensities from samples lacking Tat were subtracted from the intensities measured in samples containing Tat.

Three independent experiments were performed and data are presented as mean \pm SEM.

References

- Barret, C., Roy, C., Montcourrier, P., Mangeat, P. and Niggli, V. (2000) Mutagenesis of the phosphatidylinositol 4,5-bisphosphate (PIP(2)) binding site in the NH(2)-terminal domain of ezrin correlates with its altered cellular distribution. *J Cell Biol*, **151**, 1067-1080.
- Bessoles, S., Fouret, F., Dudal, S., Besra, G.S., Sanchez, F. and Lafont, V. (2008) IL-2 triggers specific signaling pathways in human NKT cells leading to the production of pro- and anti-inflammatory cytokines. *J Leukoc Biol*, **84**, 224-233.
- Caputo, G.A. and London, E. (2003) Using a novel dual fluorescence quenching assay for measurement of tryptophan depth within lipid bilayers to determine hydrophobic alpha-helix locations within membranes. *Biochemistry*, **42**, 3265-3274.
- Jeang, K.T., Xiao, H. and Rich, E.A. (1999) Multifaceted activities of the HIV-1 transactivator of transcription, Tat. *J Biol Chem*, **274**, 28837-28840.
- Méré, J., Morlon-Guyot, J., Bonhoure, A., Chiche, L. and Beaumelle, B. (2005) Acid-triggered Membrane Insertion of Pseudomonas Exotoxin A Involves an Original Mechanism Based on pH-regulated Tryptophan Exposure. *J Biol Chem*, **280**, 21194-21201.
- Schagger, H. (2006) Tricine-SDS-PAGE. *Nat Protoc*, **1**, 16-22.
- Stahelin, R.V., Long, F., Peter, B.J., Murray, D., De Camilli, P., McMahon, H.T. and Cho, W. (2003) Contrasting membrane interaction mechanisms of AP180 N-terminal homology (ANTH) and epsin N-terminal homology (ENTH) domains. *J Biol Chem*, **278**, 28993-28999.
- Vendeville, A., Rayne, F., Bonhoure, A., Bettache, N., Montcourrier, P. and Beaumelle, B. (2004) HIV-1 Tat enters T-cells using coated pits before translocating from acidified endosomes and eliciting biological responses. *Mol Biol Cell*, **15**, 2347-2360.
- Watt, S.A., Kular, G., Fleming, I.N., Downes, C.P. and Lucocq, J.M. (2002) Subcellular localization of phosphatidylinositol 4,5-bisphosphate using the pleckstrin homology domain of phospholipase C delta1. *Biochem J*, **363**, 657-666.
- Wessel, D. and Flugge, U.I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem*, **138**, 141-143.