Functional interaction between the cytoplasmic leucine-zipper domain of HIV-1 gp41 and p115-RhoGEF

H. Zhang*, L. Wang*, S. Kao†, I.P. Whitehead*, M.J. Hart‡, B. Liu§, K. Duus*, K. Burridge*§, C.J. Der*¶ and L. Su*†

The long cytoplasmic tail of the human immunodeficiency virus (HIV)-1 transmembrane protein gp41 (gp41C) is implicated in the replication and cytopathicity of HIV-1 [1]. Little is known about the specific functions of gp41C, however. HIV-1 or simian immunodeficiency virus (SIV) mutants with defective gp41C have cell-type- or species-dependent phenotypes [2–6]. Thus, host factors are implicated in mediating the functions of gp41C. We report here that gp41C interacted with the carboxy-terminal regulatory domain of p115-RhoGEF [7], a specific guanine nucleotide exchange factor (GEF) and activator of the RhoA GTPase, which regulates actin stress fiber formation, activation of serum response factor (SRF) and cell proliferation [8,9]. We demonstrate that gp41C inhibited p115-mediated actin stress fiber formation and activation of SRF. An amphipathic helix region with a leucine-zipper motif in gp41C is involved in its interaction with p115. Mutations in gp41C leading to loss of interaction with p115 impaired HIV-1 replication in human T cells. These findings suggest that an important function of gp41C is to modulate the activity of p115-RhoGEF and they thus reveal a new potential anti-HIV-1 target.

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Results and discussion

The gp41C domain interacts with the carboxy-terminal regulatory domain of p115-RhoGEF

To elucidate the function of gp41C, we performed a yeast two-hybrid screen to identify proteins that interact with it (see Supplementary material). From 5×10^6 cDNA clones, four clones were identified. One encodes the carboxy-terminal 60 residues (gh60) of p115-RhoGEF and the other three all encode its carboxy-terminal 53 residues (g117; Figure 1). To confirm the interaction of p115-RhoGEF with gp41C in mammalian cells, we co-expressed p115 proteins with a gp41C protein fused to six histidine residues at the amino terminus (His_6–gp41C) and labeled the cells with [^{35}S]methionine and [^{35}S]cysteine. His_6–gp41C was precipitated with Ni–NTA beads and gp41C-associated proteins were visualized by autoradiography (Figure 1b). The full-length p115 (p115FL) was coprecipitated with gp41C (lane 4), and p115FL alone was not precipitated by the Ni–NTA beads (lane 3). The p115dC protein, which lacks the carboxy-terminal 100 residues [7], was not coprecipitated with His_6–gp41C (lane 5). These results were consistent with our findings from yeast cells, in which gp41C interacted with the carboxy-terminal 53 residues of p115 (g117, Figure 1c). Like other Dbl-family Rho GEFs, p115-RhoGEF is associated with the plasma membrane through its pleckstrin homology (PH) domain [9,10]. To show colocalization of p115-RhoGEF with HIV-1 gp41, p115FL was cotransfected with the HIV-1 provirus pNL4-3 in HeLa cells. Double staining with a rhodamine-labeled anti-p115 antiserum and a fluorescein isothiocyanate (FITC)-labeled anti-gp41C monoclonal antibody showed that gp41 and p115 were both localized in the cytoplasmic membrane region (Figure 1d). Thus, gp41 may be associated with p115 at the plasma membrane.

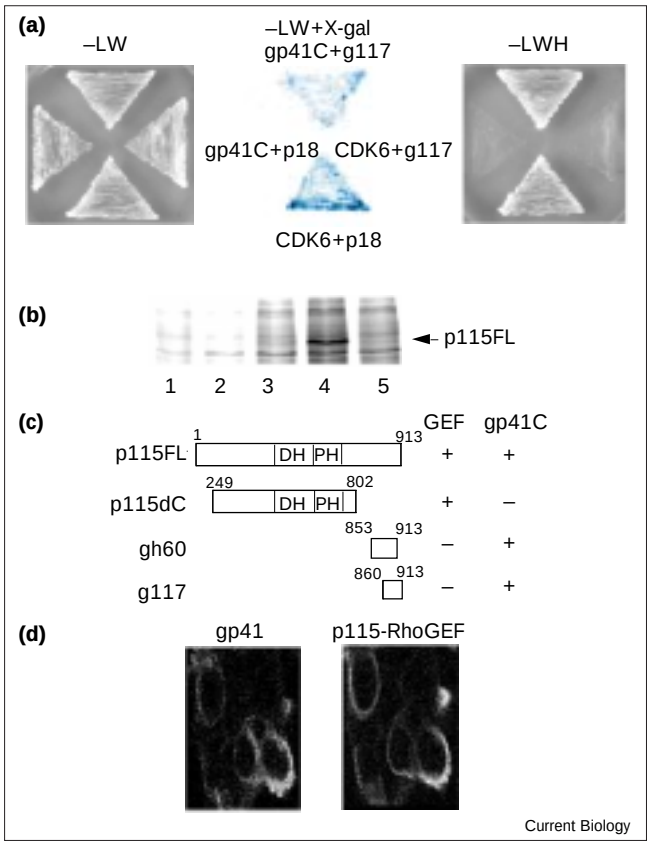
Expression of gp41C disrupts actin stress fiber organization

To test whether gp41C affected p115 activity, we studied the effect of gp41C on actin stress fiber formation in Swiss 3T3 cells [11]. Cells overexpressing p115FL or p115dC showed enhanced actin stress fiber formation and increased phalloidin staining (Figure 2a,g). Non-injected cells had low levels of actin stress fibers. Expression of gp41C led to a disruption of the actin stress fibers (Figure 2c). Co-expression of p115FL or p115dC with gp41C led to reorganized actin stress fibers (Figure 2e,g). Therefore, gp41C inhibited actin stress fiber formation. Co-expression of active p115-RhoGEF counteracted the inhibitory activity of gp41C.

Ectopic expression of gp41C inhibits p115-mediated SRF activation

We further showed that gp41C inhibited p115-mediated activation of SRF, which is activated by Rho GTPases [12]. An SRF-responsive reporter gene was activated by p115 in serum-starved NIH 3T3 cells (Figure 2i). Co-expression of gp41C inhibited this p115-mediated activation. This

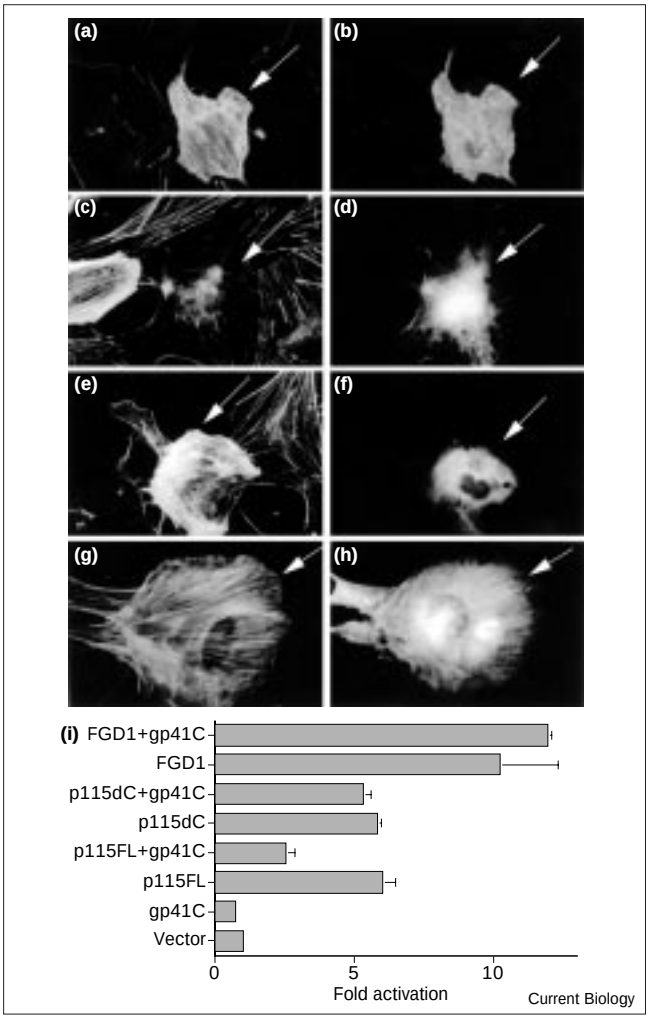
Figure 1



Interaction of gp41C with the carboxyl terminus of p115-RhoGEF. (a) Two-hybrid assay. The g117 fragment (the carboxy-terminal 53 residues of p115-RhoGEF) interacts with gp41C to activate the *his3* gene and the *lacZ* gene. CDK6 and p18-INK4c were used as positive controls. Yeast colonies that grew on plates lacking leucine and tryptophan (–LW) were tested for the expression of *lacZ* (–LW/X-gal) or on histidine-deficient plates (–LWH). (b) Coprecipitation of p115 with His_6–gp41C in transfected 293T cells. The His_6–gp41C (encoded by plasmid pcHgp41C) was pelleted with Ni–NTA beads and pelleted proteins were visualized after autoradiography. Lanes 1–5 show samples from cells transfected with pcDNA3 vector alone (lane 1), pcHgp41C and pcDNA3 (lane 2), p115FL and pcDNA3 (lane 3), pcHgp41C and p115FL (lane 4), or pcHgp41C and p115dC (lane 5). Both p115FL and p115dC were efficiently expressed (data not shown). (c) Schematic representation of the structure and function of the p115 derivatives. Protein p115FL, full-length p115; p115dC, a truncated p115 fragment (residues 249–802); gh60 and g117, the gp41C-interacting fragments isolated from the two-hybrid assays (residues 853 (gh60) or 860 (g117) to 913 of p115-RhoGEF). GEF, guanine nucleotide exchanging activity on RhoA [7]; gp41C, interaction with the gp41C protein (in yeast and 293T cells); DH, Dbl homology region; PH, pleckstrin homology region. (d) Colocalization of gp41 and p115-RhoGEF. HeLa cells were cotransfected with DNAs encoding p115 and HIV-1 provirus NL4-3. Cells were stained with an anti-p115 antiserum [7] and an anti-gp41 monoclonal antibody. Secondary antibodies (rhodamine-labeled goat anti-rabbit and FITC-labeled anti-mouse) were used to detect p115 and gp41, respectively. Pre-immune or isotype controls showed no significant staining (data not shown).

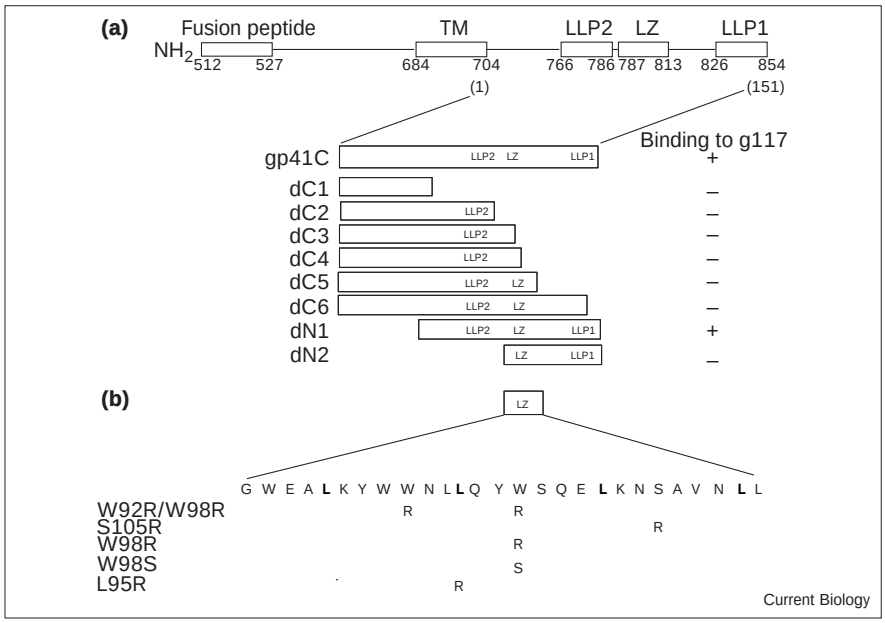
inhibition depended on the interaction between gp41C and p115 because p115dC, which did not interact with

Figure 2



Inhibition of p115-RhoGEF-mediated RhoA activation by gp41C. (a–h) Disruption of actin stress fiber organization by gp41C. Swiss 3T3 cells were micro-injected with plasmid DNA and serum-starved for 12–16 h before fixation and staining of actin stress fibers. (a,b) Cells injected with Myc-tagged p115FL alone; (c,d) cells injected with a plasmid encoding enhanced green fluorescent protein under the control of the CMV promoter (pCMV−eGFP) and pcHgp41C; (e,f) cells injected with p115FL and pcHgp41C; (g,h) cells injected with Myc-tagged p115dC and pcHgp41C. The injected cells (arrows) were identified either (d) by GFP signals or (b,f,h) by immunostaining of the Myc epitope-tagged p115 proteins. The rhodamine–phalloidin-stained stress fibers were visualized in (a,c,e,g). Representatives of about 20 microinjected cells for each sample are shown. Two independent experiments were performed. (i) Inhibition of p115-RhoGEF-mediated activation of SRF by gp41C. A plasmid with a luciferase gene controlled by a mutant c-Fos serum response element (SREm2-Luc) that no longer responds to ternary complex factor (TCF) and measures only SRF activity (see Supplementary material) was co-expressed with plasmids encoding fragments of gp41 and/or p115, and luciferase activity was measured. Co-expression of gp41C inhibited p115FL-mediated activation by 70% (p115+gp41C), but did not inhibit p115dC-mediated activation (p115dC+gp41C) or FGD1-activated SRF (FGD1+gp41C). The experiments were performed in triplicate and repeated three times. Error bars indicate standard deviations.

Figure 3



A putative leucine-zipper (LZ) motif of gp41C is involved in the interaction with p115. (a) Mapping of the gp41C domains involved in interaction with the carboxyl terminus of p115-RhoGEF. TM, transmembrane domain; LLP1 and LLP2, lentivirus lytic peptides. The numbers 1–854 indicate residue positions in the HIV-1 gp160 env polypeptide. The residues after the TM domain (704–854) are referred to as gp41C and numbered 1–151. The fragments gp41C (1–151), dC1 (1–47), dC2 (1–70), dC3 (1–90), dC4 (1–97), dC5 (1–108), dC6 (1–139), dN1 (53–151), and dN2 (83–151) were fused to the Gal4 DNA-binding domain. Binding to g117 (+ or –) was determined by growth on His$^-$ plates and expression of *lacZ*, as in Figure 1. (b) Mutations defined by the reverse yeast-two-hybrid screen are localized around the LZ region. The amphipathic α-helix region containing the LZ motif is shown in the single-letter amino acid code and the leucines comprising the LZ motif ($LX_6LX_6LX_6L$, where X is any amino acid) are in bold. The mutations defined by the reverse two-hybrid screen leading to loss of interaction with g117 are marked.

gp41C, also activated the SRF reporter but gp41C co-expression failed to inhibit its activity (Figure 2i). Furthermore, gp41C showed no inhibition of SRF activation mediated by other Rho GEFs such as FGD1 [13] (Figure 2i) and Dbl (data not shown). Therefore, gp41C interacted with p115-RhoGEF to inhibit its activity. Further investigation of the interaction between gp41C and p115 should help to elucidate the mechanism of GEF-mediated Rho activation.

Inhibition of p115-mediated activation of RhoA by gp41C has significant implications for HIV-1 pathogenesis. First, inhibition of RhoA activity has been reported to enhance the spreading and migration of monocytes and macrophages [14]. Thus, gp41C may interact with p115-RhoGEF to modulate target-cell migration from the initial site of infection. Second, RhoA is also involved in regulating cell survival and cell-cycle progression [15,16]. Inhibition of p115-RhoGEF (or RhoA) activity by gp41C may lead to depletion of human T cells and progenitor cells [17,18].

A putative leucine-zipper motif in gp41C is involved in the interaction with p115-GEF

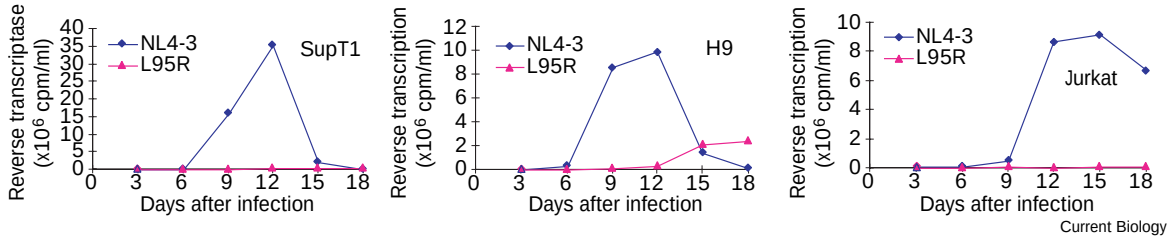
Several structural motifs implicated in protein–protein interaction and signal transduction are present in gp41C (Figure 3a). Two lentivirus lytic peptides (LLP1 and LLP2) interact with calmodulin [19] and inhibit Ca^{2+}-dependent T-cell activation [20]. A leucine-zipper motif (790–811) has recently been shown to bind lipid membranes [21]. To map the region of gp41C required for the interaction with p115, gp41C was dissected by deletion analysis (Figure 3a). Deletion of 12 or more residues at the carboxyl terminus (dC1–dC6) abolished interaction with p115 (g117). Deletion of the amino-terminal 52 residues of gp41C (dN1), however, had no effect on the interaction. When the LLP2 sequences were deleted (dN2), interaction with p115 was lost. Thus, the carboxy-terminal 98 residues of gp41C, containing the two LLPs and the leucine-zipper motif, are necessary and sufficient to mediate interaction of gp41C with p115.

To define specific residues in gp41C involved in direct interaction with p115, a modified 'reverse' two-hybrid assay (see Supplementary material) was performed. Four out of five mutants identified carried mutations in the amphipathic helix region of gp41C that encodes the putative leucine-zipper motif (Figure 3b). The fifth mutation was in the LLP1 region (data not shown). The four leucine-zipper mutations (tryptophan to serine or arginine and serine to arginine) resulted in an increase in charge on the hydrophobic side of the amphipathic helix. To further confirm that the leucine-zipper motif is involved, we introduced an arginine replacing the second leucine in the leucine-zipper motif (L95R), which also led to loss of interaction with p115.

Loss of interaction between gp41C and p115-RhoGEF impaired HIV-1 replication

To prove genetically the importance of the interaction between gp41C and p115, the L95R mutation was introduced into the HIV_{NL4-3} genome. The HIV-1 NL4(L95R)

Figure 4



The interaction between gp41C and p115-RhoGEF correlates with HIV-1 replication in human T-cell lines. SupT1, H9 or Jurkat cells were infected with an equal number of infectious units of NL4-3 or NL4(L95R) mutant viruses. Viral replication (as measured by reverse transcriptase activity) was monitored every 3 days for 18 days after infection. The experiments were repeated three times with similar results.

mutant produced wild-type levels of infectious virions when transfected into 293T cells (data not shown). When human leukemia T cells (SupT1, H9, and Jurkat cells) were infected, the L95R mutant had impaired replication (Figure 4). Therefore, the interaction of gp41C with p115 correlated with enhanced HIV-1 replication in human T-cell lines. This interaction provides a new host target (p115-RhoGEF) and a new viral target (gp41C) for the development of anti-HIV therapies.

Supplementary material

Supplementary material including additional experimental details is available at http://current-biology.com/supmat/supmatin.htm.

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Supplementary material

Functional interaction between the cytoplasmic leucine-zipper domain of HIV-1 gp41 and p115-RhoGEF

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Supplementary materials and methods

Reagents, plasmids and cell lines

The cDNA encoding the cytoplasmic domain of gp41 (the carboxy-terminal 150 residues) was cloned into the yeast two-hybrid 'bait' vector (pGBT8, ClonTech) such that it was fused in frame with the DNA binding domain of Gal4, to generate pGBT8-gp41C [S1]. Sequences encoding His–gp41C, p115FL, and p115dC were all cloned into the pcDNA3 mammalian expression vector (InVitrogen). The *lacZ*, *FGD-1*, and *Dbl* cDNAs were cloned in the pAX142 mammalian expression vector [S2]. The luciferase gene controlled by a mutant c-Fos serum response element (SREm2-Luc) that no longer responds to TCF and measures only SRF activity was described previously [S3].

The 293T and HeLa cell lines (American Type Culture Collection, ATCC) were maintained in DMEM supplemented with 10% FBS and NIH 3T3 and Swiss 3T3 cells were cultured in DMEM supplemented with 10% newborn calf serum. Cells were seeded on six-well plates (2×10^5 cells per well) the day before transfection or microinjection. Jurkat, SupT1 (ATCC), and H9 cells were maintained in RPMI-1640 supplemented with 10% FBS.

Yeast two-hybrid screening

Plasmid pGBT8-gp41C was used as a bait to screen a human thymus-derived and HeLa-derived yeast two-hybrid library (ClonTech). About 5×10^6 independent colonies were screened, and 187 colonies that grew on His-deficient plates were further screened for their β-galactosidase production. Four clones were chosen for further analysis because they encoded peptides that interacted strongly with gp41C to activate both the *His* and *lacZ* reporter genes. Positive clones were first analyzed by PCR to determine the size of the insert. The expected clones were then recovered from yeast cells by DNA extraction and transformation into *E. coli* (Matchmaker, ClonTech). The putative clones were further confirmed by cotransfecting with the pGBT8-gp41C bait plasmid in yeast cells. PCR products from the recovered plasmids were sequenced by the dideoxynucleotide termination method (Lineberger Comprehensive Cancer Center sequencing facility, University of North Carolina at Chapel Hill). The BLAST program was used to search the DNA databases.

Reverse yeast-two-hybrid screening

The gp41C region was mutagenized by PCR as described [S4]. Briefly, the dTTP concentration was limited so that about one misincorporation of nucleotides (dGTP, dATP or dCTP) was introduced for each gp41C PCR product. The PCR products were cotransformed with the pGBT8 vector, cut in the cloning region, into the yeast Y7 strain stably expressing the g117 (p115) prey. Gap repair activity in yeast ligated the mutagenized gp41C fragments into the pGBT8 vector [S4]. The yeast colonies were screened for their growth on $Leu^-Trp^-His^-$ plates and their expression of *lacZ* was analyzed using a β-galactosidase assay. The $His^-/lacZ^-$ mutants were confirmed and sequenced to identify the mutations that led to the loss of interaction.

Coprecipitation assays

A plasmid encoding His–gp41C (pcHgp41C) was cotransfected with vector DNA or plasmid DNA encoding p115-RhoGEF (p115FL or p115dC) into 293T cells with Lipofectamine (Gibco BRL). In each transfection with 4 μl of the Lipofectamine reagent, 2 μg of total plasmid DNA (1 μg for each plasmid) was used. The pcDNA3 vector was used in transfections where only one effector plasmid was included. Cells were radiolabeled with [^{35}S]methionine and [^{35}S]cysteine (80 μCi per well, Dupont NEN) for 16 h (from 32 h to 48 h after transfection). Cell extracts were prepared by lysis of cells (5×10^6 per well) in 0.5 ml of lysis buffer (0.1% Triton X-100, 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM $NaVO_3$) with protease inhibitors (0.1 mM each PMSF, aprotinin, leupeptin, and pepstatin) on ice for 30 min, followed by 10 min centrifugation in a microcentrifuge ($12{,}000 \times g$) at 4°C. His–gp41C protein was directly pelleted with Ni–NTA-beads (KIAGEN). Precipitated proteins were analyzed by SDS–PAGE followed by direct autoradiography.

Immunostaining of p115-RhoGEF and HIV-1 gp41

HeLa cells were cotransfected with DNAs encoding p115 and HIV-1 provirus NL4-3 [S5]. Cells were processed at 40 h after transfection with an anti-p115 antiserum [S6] and an anti-gp41 monoclonal antibody (kindly provided by the NIH AIDS Research and Reagent Program). Secondary antibodies (rhodamine-labeled goat anti-rabbit and FITC-labeled anti-mouse) were used to detect p115 and gp41, respectively. Immunofluorescence staining and photography were performed as described [S7].

Actin stress fiber staining

Swiss 3T3 cells were micro-injected with pcHgp41C (and/or a plasmid encoding Myc-tagged p115) and pCMV–GFP. After incubation for 18 h in serum-free DMEM, the injected cells were analyzed by immunostaining for actin stress fibers and Myc–p115 essentially as described [S8]. Briefly, cells were fixed in formaldehyde (3.7% in PBS) for 10 min and permeabilized in Triton X-100 (0.5%) for 6 min. They were then incubated with anti-Myc antibody, followed by FITC-conjugated goat anti-mouse immunoglobulin G and rhodamine-conjugated phalloidin (Molecular Probes). Injected cells were visualized by standard fluorescence microscopy for GFP, rhodamine (for actin stress fibers) or FITC (for Myc).

Rho-dependent SRF reporter assays

Briefly, plasmids encoding p115 (or p115dC, FGD-1 or Dbl), gp41C, and SREm2-Luc were transfected into NIH 3T3 cells. The cells were serum-starved from 24 h to 36 h after transfection and a luciferase assay was performed using enhanced chemiluminescent reagents and a Monolight 2010 Luminometer (Analytical Luminescence). All assays were performed in triplicate and repeated at least three times. The internal control reporter plasmid pAX142-lacZ was included in the transfection mix and all values were normalized relative to β-galactosidase activity. No significant difference in β-galactosidase activity was observed between different samples.

Construction of NL4(L95R) gp41 mutant

To construct an infectious NL4-3 proviral clone containing the L95R mutation in gp41, primer L95RF (5′-TGGGAAGCCCTCAA ATATTGGTGGAATCTCCGACAGTATTGGAGTCAG-3′, nucleotides 8590–8637; the mutated nucleotide is in bold) and L95RR (5′-CTGACTCCAATACTGTCGGAGATTCCACCAATATTTGAGGGC TTCCCA-3′, nucleotides 8367–8590) were used to perform site-directed mutagenesis in the plasmid pBS.HIV/NL4-3.SalI/XbaI using the QuikChange site-directed mutagenesis kit (Stratagene). Plasmid pBS-NL4-SalI/XbaI is a pBS (Stratagene)-based vector containing the *Sal*I-*Xho*I fragment of NL4-3 [S5]. The mutated *Bam*HI–*Xho*I fragment was sequenced and cloned into the NL4-3 proviral DNA clone. The

resulting proviral clone (pNL/L95R) was sequenced to confirm the mutation. The Rev protein encoded by the other reading frame was not altered.

HIV-1 viral stock production and virus-replication assays

The pNL/L95R mutant or pNL4-3 plasmid was transfected into 293T cells. Virus production was measured with a reverse transcriptase assay and an infectious-units assay on MAGI cells [S9]. HIV-1 replication was performed as described [S10]. Briefly, about 3,000 infectious units of NL4-3 or L95R mutant were used to infect 0.5×10^6 T cells (SupT1, Jurkat or H9). HIV-1 replication was monitored every three days by reverse transcriptase assay in culture medium.

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