The PDZ-adaptor protein syntenin-1 regulates HIV-1 entry

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Abstract

Syntenin-1 is a cytosolic adaptor protein involved in several cellular processes requiring polarization. Human immunodeficiency virus type 1 (HIV-1) attachment to target CD4+ T-cells induces polarization of the viral receptor and coreceptor, CD4/CXCR4, and cellular structures toward the virus contact area, and triggers local actin polymerization and phosphatidylinositol 4,5-bisphosphate (PIP2) production, which are needed for successful HIV infection. We show that syntenin-1 is recruited to the plasma membrane during HIV-1 attachment and associates with CD4, the main HIV-1 receptor. Syntenin-1 overexpression inhibits HIV-1 production and HIV-mediated cell fusion, while syntenin depletion specifically increases HIV-1 entry. Down-regulation of syntenin-1 expression reduces F-actin polymerization in response to HIV-1. Moreover, HIV-induced PIP2 accumulation is increased in syntenin-1–depleted cells. Once the virus has entered the target cell, syntenin-1 polarization toward the viral nucleocapsid is lost, suggesting a spatiotemporal regulatory role of syntenin-1 in actin remodeling, PIP2 production, and the dynamics of HIV-1 entry.

Introduction

The adaptor protein syntenin-1 was originally described in relation to the syndecan receptor and its recycling. This article was published online ahead of print in MBoC in Press (http://www.molbiocell.org/cgi/doi/10.1091/mbc.E11-12-1003) on April 25, 2012. Address correspondence to: Francisco Sánchez-Madrid (fsanchez.hlpr@salud.madrid.org).

Abbreviations used: APC, antigen presenting cell; BSA, bovine serum albumin; CD4, cluster of differentiation-4; CMTMR, 5-(and-6)((4-chloromethyl)benzoyl)amino)tetramethylrhodamine; CNBr, cyanogen bromide; CXCR4, C-X-C chemokine receptor type-4; ELISA, enzyme-linked immunosorbent assay; ENV, viral envelope glycoprotein complex gp120/gp41; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; HIV-1, human immunodeficiency virus type 1; ITAM, immunoreceptor tyrosine-based activating motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; mda-9, melanoma differentiation-associated gene-9 (mda-9) for its up-regulated expression in several types of tumors, which correlates with high invasiveness (Sarkar et al., 2004, 2008). Structurally, syntenin-1 contains a regulatory N-terminal region and two tandem postsynaptic density protein, Drosophila disk large, and zona occludens-1 (PDZ) domains. The N-terminal region is composed of two Tyr-based domains: one ITIM and one ITAM domain, the ITIM domain being important for CXCR4-mediated T-cell migration (Sala-Valdes et al., 2012). PDZ domains are involved in the formation of macro-molecular complexes that often link transmembrane proteins to the actin cytoskeleton, mediating their clustering and polarized subcellular distribution (Fanning and Anderson, 1999; Brone and Eggermont, 2005; Hirbec et al., 2005; Ludford-Menting et al., 2005). PDZ domains interact with short amino acid sequences at the C-terminal end of plasma membrane proteins. Three consensus sequences bind PDZ domains: class I ([S/T]-X-Φ), class II (Φ-X-Φ), and class III ([D/E]-X-Φ), where Φ is any amino acid and Φ is a hydrophobic residue (Chimura et al., 2011). Through its PDZ domains, syntenin-1 binds to transmembrane proteins, such as CD6 (Gimferrer et al., 2005) and the tetraspanin CD63 (Latsycheva et al., 2006); signaling proteins, such as the tyrosine kinase Src (Boukerche et al., 2008);...
and the actin-linker protein merlin (Jannatipour et al., 2001). Recruitment of synteny-1 to the plasma membrane is also regulated through its PDZ-mediated interaction with the lipid second messenger phosphatidylinositol 4,5-bisphosphate (PIP$_2$; Zimmermann, 2006).

Syntenin-1 has a cytosolic distribution, being enriched in vesicles and F-actin structures (Zimmermann et al., 2001). It is implicated in several actin-polarized processes, such as cell migration, immune synapse formation, intracellular trafficking, cell-surface targeting, axonal outgrowth, and synaptic transmission (Gimmferrer et al., 2005; Hirbec et al., 2005; Zimmermann et al., 2005; Beekman and Coffer, 2008; Sarkar et al., 2008). We recently reported the involvement of synteny-1 in CXCR4-mediated T-chemotaxis and T-APC antigen presentation, in which it regulates Rac-triggered actin polymerization (Sala-Valdes et al., 2012).

CD4/CXCR4-derived signaling and Rac-induced actin remodeling are also important for human immunodeficiency virus type 1 (HIV-1) infection (Pontow et al., 2004; Carter et al., 2009). Soon after HIV-1 contact with CD4$^+$ T-cells, the viral envelope glycoprotein complex gp120/gp41 (Env) engages with CD4 and CXCR4, inducing several signaling pathways that trigger receptor clustering (Jimenez-Baranda et al., 2007; Barrero-Villar et al., 2009) in a structure called “cap” (Dianzani et al., 1995; Nguyen et al., 2005), local actin polymerization (Iyengar et al., 1998; Jolly et al., 2004), T-cell activation (Popik et al., 1998; Hiscott et al., 2001), and HIV-1 replication (Popik and Pitha, 2000; Mettling et al., 2008). These rearrangements enhance viral entry (Wu and Yoder, 2009; Juno and Fowke, 2010) by supplying an optimal density of CD4/CXCR4 complexes through actin-mediated surface reorganization (Iyengar et al., 1998; Doms, 2000; Jolly et al., 2004). Thus the actin-binding proteins moesin and filamin-A accumulate at the F-actin–enriched capping areas and influence CD4/CXCR4 clustering (Jimenez-Baranda et al., 2007; Barrero-Villar et al., 2009). Moreover, we previously showed that HIV-1 gp120, through its interaction with CD4, induces local production of PI$_2$ by activating PI4PSK-1x (Barrero-Villar et al., 2008).

After viral attachment to the plasma membrane, the local F-actin accumulation can constitute a physical barrier for entry of the viral nucleocapsid (Vasiliver-Shamis et al., 2009). In this regard, CXCR4-gp120 interaction was recently shown to activate cofilin, which promotes actin clearance, facilitating nucleocapsid entry into the cytoplasm (Yoder et al., 2008).

In this study, we assessed the possible role of synteny-1 as a mediator of CD4/CXCR4 clustering and actin remodeling during HIV-1 infection. Our data indicate that synteny-1 associates with CD4 and negatively regulates HIV-1 infection by affecting the dynamic reorganization of actin cytoskeleton and PI$_2$ production triggered upon HIV-1 contact, specifically at the viral entry step.

RESULTS

**Syntenin-1 is recruited to the plasma membrane by HIV-1 Env**

PDZ proteins commonly determine the polarization of cellular events (Fanning and Anderson, 1999; Brone and Eggermont, 2005; Ludford-Menting et al., 2005). Because HIV-1 contact with target cells triggers the polarization of cellular components (Dianzani et al., 1995; Nguyen et al., 2005), we investigated whether the PDZ protein synteny-1 was recruited upon HIV-1 contact with target CD4$^+$ T-cells (CEM-T4). In the absence of HIV-1 virus, synteny-1 displayed a diffuse cytosolic pattern with low clustering at the plasma membrane (Figure 1A). In contrast, after incubation of T-cells with free HIV-1 particles, synteny-1 was clearly recruited toward the CD4/CXCR4 capping area (Figure 1, A and E). To confirm the specificity of synteny-1 recruitment, we assessed CD45 distribution and observed that it did not accumulate at virus-induced capping areas (Figure 1, B and E). In addition, to assess that synteny-1 recruitment was Env-mediated, target CEM-T4 cells were incubated with a Jurkat clone expressing the T-tropic HIV-1 envelope HxB (Env$^+$ HxBc2 cells). Syntenin-1 was found at the Env-driven cell contacts, together with CD4 (Figure 1, C and E). We also cocultured target CEM-T4 cells with T-cells directly infected with HIV–green fluorescent protein (GFP) virus (MT4-HIV-GFP). In these cocultures, synteny-1 accumulated at cell–cell contacts, areas in which viral synapses are established (Figure 1, D and E), but not at unspecific Env-independent cell–cell contacts (Figure 1E, right-hand white bar; exemplified in Supplemental Figure S1). Quantification confirmed significant accumulation of synteny-1 at contacts with HIV-1 viral particles and infected cells, in comparison with CD45 (Figure 1E).

**Syntenin-1 associates with the cytoplasmic tail of CD4**

PDZ domains usually bind to specific consensus sequences at the C-terminal ends of transmembrane proteins (Chimura et al., 2011). To determine whether synteny-1 forms part of the CD4/CXCR4 complex at the capping areas, we first searched the CD4 and CXCR4 cytoplasmic domain sequences for potential PDZ-binding motifs. A class I PDZ-binding consensus sequence (Ser-Pro-ile) was detected at the C-terminal end of CD4, suggesting potential direct interaction with synteny-1 via its PDZ domains. To test this, we immobilized glutathione S-transferase (GST)-fused hemagglutinin (HA)- or Myc-tagged synteny-1 on Sepharose beads, and incubated them with human primary lymphoblast lysates. GST-syntenin-HA and GST-syntenin-Myc, but not GST alone, precipitated CD4 (Figure 2A). Confirming this interaction, anti-CD4 antibody immunoprecipitated endogenous synteny-1 from CEM T-cell lysates; synteny-1 was detected with anti-syntenin-1 antibody as a double band of around 32 kDa (Figure 2B). To demonstrate the occurrence of CD4–syntenin-1 association in the context of HIV-1 infection, we performed reverse immunoprecipitation assays with Jurkat T-cells previously incubated with free viral particles. Immunoprecipitation of synteny-1, followed by immunodetection of CD4, detected constitutive CD4–syntenin-1 interaction that was maintained during HIV-1 contact (Figure 2C). Together, these data suggest that synteny-1 interacts with CD4 before and during HIV-1 infection.

**Syntenin-1 overexpression inhibits HIV-1–induced cell fusion and viral production**

To assess the specific role of synteny-1 during HIV-1 infection, target T-cells were transfected with GFP-tagged, wild-type synteny-1 (syntenin-GFP) or a mutated protein, synteny-Y1-GFP (Synt-Y1-GFP), which carries a Y4>F point mutation in the ITIM motif of the regulatory N-terminal region. Phosphorylation of endogenous synteny-1 at this tyrosine residue has been shown to be essential for CXCR4-induced chemotaxis (Sala-Valdes et al., 2012). Syntenin-GFP and Synt-Y1-GFP showed a similar distribution to endogenous synteny-1, being enriched at Env-driven cell–cell contacts (Figure 3A) and HIV-induced capping areas (Figure 3B and data not shown). Target J77 cells overexpressing these GFP-tagged proteins were infected with free HIV-1 particles, and viral production was measured 3 d later by p24 enzyme-linked immunosorbent assay (ELISA). Cells overexpressing synteny-GFP produced lower viral titers than control cells (transfected with GFP alone). In contrast, overexpression of the mutated synteny-1 had no effect on HIV-1 production (Figure 3C).

To investigate the potential effect of synteny-1 plasma membrane recruitment on HIV-1–induced cell fusion, we measured Env-mediated cell fusion by flow cytometry. Env$^+$ HxBc2 cells
were labeled with red intracellular tracker (CMTMR; 5-(and-6)-(4-chloromethyl)benzoyl)-amino) tetrachloromethylrhodamine) and cocultured with target T-cells transfected with each GFP-tagged syntenin-1 construct. Env-driven cell fusion leads to syncytia formation, detected as double-fluorescence positive events of higher size and complexity. As a control of specificity, syncytia formation was blocked with the HIV-1 fusion inhibitor T-20 (Figure 3D). Quantification of these events showed that overexpression of wild-type syntenin-1 reduced Env-mediated cell fusion, whereas the Y4>F mutant had no significant effect (Figure 3D). These data suggest that syntenin-1 negatively regulates HIV-1–mediated membrane fusion through a mechanism requiring phosphorylation of tyrosine 4 in the ITIM motif. To assess whether the Y4>F point mutant was able to interact with CD4, cells were transfected with syntenin-GFP or Synt-Y1-GFP, immunoprecipitated with an anti-GFP, and blotted for CD4. As shown in Figure 3E, Synt-Y1-GFP also associated with CD4.

Knockdown of syntenin-1 expression increases HIV-1 cell fusion and viral entry

To further study syntenin-1 function during HIV-1 infection, we knocked down endogenous syntenin-1 expression using small interfering RNA (siRNA). Control cells were transfected with an siRNA sequence that does not hybridize with any eukaryotic mRNA. Syntenin-1 down-regulation was assessed for each experiment (Figure 4A and data not shown). Membrane expression levels of CD4 and CXCR4 were also routinely assessed to confirm that they were not affected by syntenin depletion (Figure 4B). To assess Env-driven cell fusion, silenced T-cells loaded with green cell tracker were cocultured with HIV-1–infected T-cells (MT4-HIV-GFP) and immunostained for CD4 (CD4v4-FITC) and CD45 (D3/9). Projections of confocal stack images are shown. Scale bar: 5 μm. Surface plots show double-fluorescence distribution and intensity for corresponding images. (D) Target J77 cells were incubated for 2 h with HIV-1–infected T-cells (MT4-HIV-GFP) and immunostained for syntenin-1 and CD4 (HP2/6). Projections of confocal stack images are shown. Scale bar: 5 μm. (E) Quantification of syntenin-1 accumulation at HIV-1-induced capping areas (HIV-wt), contacts between target cells and HIV-infected cells (MT4-J77), or contacts between target cells and Env* cells (HxB-J77). The control (white bars) for HIV wild-type and MT4-J77 represent the recruitment of CD45. The control (white bar) for HxB-J77 corresponds to the recruitment of syntenin at Env-independent cell–cell contacts (between two target J77 cells). The chart represents the mean ± SD of three independent experiments; more than 100 conjugates were counted in each condition.
Syntenin-1 restricts HIV-1 entry

Syntenin-1 recruitment at the plasma membrane suggested its involvement during HIV-1 attachment or entry steps. To distinguish between these possibilities, we incubated syntenin-1–depleted cells with HIV-1 particles for 2 h at 4°C, a temperature that does not allow Env-induced membrane fusion, thus arresting the virus at the attachment step, or at 37°C, a permissive temperature for membrane fusion and consequently for viral entry. For both conditions a low infection rate was used to avoid superinfection and cooperative virion entry. The amount of p24 in the lysates was measured by ELISA (Figure 5A) and Western blot (data not shown); HIV-1 attachment was estimated from the p24 level at 4°C, and HIV entry was estimated from the difference between the levels at 37 and 4°C. Syntenin-1 siRNA knockdown for 3 days significantly reduced HIV-1 infection (Figure 5B). VSV infection was similar in silenced and control cells, demonstrating that the effect observed is HIV-1 Env-dependent (Figure S2). These results indicate that syntenin-1 negatively regulates HIV-1 entry without affecting viral attachment.

To study the behavior of syntenin-1 during viral attachment and entry in more detail, we incubated Gag-GFP fluorescent virus-like particles (VLPs) with T-cells at 4°C or 37°C. At 4°C, VLPs attached preferentially to one pole of the target cell (Figure S3; see Supplemental Movie S1 for three-dimensional reconstruction), which suggests that the capping area behaves as the main site for viral entry, as has been proposed (Doms, 2000; Nguyen et al., 2005). At 37°C, VLPs could be observed inside target cells, usually accumulated at one pole of the cytoplasm (Figure S3; see Movie S2 for three-dimensional reconstruction).

We next examined the localization of endogenous syntenin-1 by confocal immunostaining of T-cells exposed to VLPs. To avoid false positives due to fortuitous superposition, we only analyzed planes in which VLPs were detected (usually one to two planes distanced by 0.25 μm). Syntenin-1 was enriched at VLP attachment sites in cells kept at 4°C (Figure 5, C and F), whereas barely any syntenin-1 localized at the internalized VLPs in cells incubated at 37°C (Figure 5, D and F). However, syntenin-1 did accumulate near VLPs positioned at cell–cell contacts at 37°C (Figure 5, E and F). Quantification of syntenin-1 accumulation at VLPs at each temperature and location confirmed that association of syntenin-1 with VLPs occurs only on the plasma membrane, and not once the particles are already internalized (Figure 5F).

Thus syntenin-1 is recruited to the plasma membrane by HIV-1 Env during the attachment and entry steps but affects only the effectiveness of viral entry, and it is released from the viral complex once the nucleocapsid has entered the cytoplasm.

Syntenin-1 regulates actin polymerization and PIP₂ accumulation upon HIV-1 contact

Syntenin-1 was recently found to regulate F-actin polymerization via Rac GTPase in T-cells during CXCR4-induced chemotaxis and superantigen presentation (Sala-Valdes et al., 2012). This syntenin-1–mediated activation of Rac depends on phosphorylation of tyrosine 4 (Sala-Valdes et al., 2012). Moreover, Rac-mediated actin reorganization is known to be essential for HIV-1 entry (Iyengar et al., 1998; Pontow et al., 2004; Harmon et al., 2010). To analyze the relation between syntenin-1 and F-actin during HIV-1 infection, we incubated syntenin-1–depleted cells with VLPs, stained them for syntenin-1 and F-actin, and analyzed them with confocal microscopy. Syntenin-1 clusters at the plasma membrane corresponded with the VLP contact area and partially overlapped with F-actin accumulation (Figure 6A; see Figure S4 for F-actin staining in noninfected target cells). Given that HIV-1 attachment to CD4 and CXCR4 triggers local actin polymerization (Jolly et al., 2004), we analyzed this process in syntenin-1–depleted cells. A similar percentage of VLPs colocalized with F-actin in control and syntenin-1–depleted cells (Figure 6A). However, when actin polymerization after HIV-1 contact was monitored at different time points, control cells showed a marked increase in F-actin content 30 min after HIV-1 contact, whereas F-actin polymerization was markedly lower in syntenin-1–depleted cells (Figure 6B). These data indicate that syntenin-1 is required for HIV-1–triggered F-actin polymerization at early stages, when most virus particles are observed at the plasma membrane. Moreover, F-actin accumulation at cell–cell contacts between target and infected MT4 cells was reduced in cells knocked down for syntenin in comparison with control cells (Figure 6C).
Interaction of the HIV-1 envelope with CD4 triggers PIP₂ production (Barrero-Villar et al., 2008), and this lipid has been found to regulate not only syntenin-1 recruitment to the plasma membrane (Zimmermann et al., 2005) but also many other actin-related proteins (Saarikangas et al., 2010). We therefore assessed whether syntenin-1–dependent actin polymerization upon HIV-1 contact was related to local production of PIP₂. Control or syntenin-1–depleted cells were incubated with MT4-HIV-GFP cells for 2 h and then fixed and stained for PIP₂ with anti-PIP₂ mAb. Syntenin-1–depleted T-cells showed increased accumulation of PIP₂ at contacts with HIV-1–infected T-cells (Figure 6D). To confirm that this effect was related to HIV-1 Env-dependent contacts, we quantified PIP₂ accumulation in target cells
To assess whether syntenin-1 depletion increases PI(4,5)P₂ accumulation in virus–cell contacts, we transfected control and syntenin-1–depleted cells with PLCδ3-PH-GFP (pleckstrin homology domain of PLCδ3-tagged to GFP), a biosensor that binds to PI(4,5)P₂-enriched membranes (Varnai and Balla, 1998), and incubated them with Cherry-fluorescent VLPs expressing HxB (T-tropic) envelope. Patches of PLCδ3-PH-GFP were frequently observed on the target cell surface at sites of VLP attachment (a representative image is shown in Figure 6F). The frequency of these events was higher in syntenin-1–depleted cells (Figure 6F). Taken together, these data indicate that syntenin-1 depletion increases PI(4,5)P₂ accumulation at Env-driven contacts both of cell–cell and of virus–cell interactions, correlating with lower F-actin polymerization and higher viral entry.

FIGURE 4: Knockdown of syntenin-1 expression increases HIV-1 entry and syncytia formation. (A) Representative Western blot showing silencing of endogenous syntenin-1 expression using oligonucleotide J-008270-08 in T-cells 48 h after siRNA transfection. Numbers below the blot show band intensity ratios between cells transfected with control siRNA and syntenin-1 siRNA, expressed relative to tubulin. (B) Bar chart showing surface expression levels (mean fluorescence intensity) of CD4 and CXCR4 in control and syntenin-depleted cells measured by flow cytometry. (C) Syntenin-1 silencing in target T-cells favors syncytia formation induced by Env⁺ HxBc2 cells. Syncytia formation was quantified by flow cytometry detection of double-positive fluorescent cells and is presented as the fold induction relative to cells transfected with control siRNA (mean ± SD of four independent experiments performed in duplicate; p = 0.017). As a control, syncytia formation was blocked by incubation with the fusion inhibitor T20 (10 nM). (D) Effect of syntenin-1 silencing on HIV-1 infection. Infection was measured as p24 viral protein content in supernatants (ELISA) and depicted as the mean fold induction relative to cells transfected with control siRNA (mean ± SD of four experiments performed in triplicate; p = 0.025). (E) Syntenin-1-GFP overexpression on cells knocked down for the endogenous syntenin-1 rescues the level of HIV-1 infection. Infection was measured as p24 viral protein content in supernatants (ELISA) and quantified as the mean fold induction relative to cells transfected with control siRNA + GFP (mean ± SD of two experiments performed in triplicate).

cocultured with Env⁺ HxBc2 cells (heterotypic, Env-driven contacts) or cultured alone (homotypic, Env-independent contacts between target cells; Figure 6E and see Figure S5 for an example image). Syntenin-1 knockdown specifically increased PI(4,5)P₂ accumulation at Env-driven contacts, while having no effect on Env-independent contacts (Figure 6E).
cofilin activation and its actin-severing function have been linked to enhanced HIV entry (Yoder et al., 2008). Thus a reduced F-actin structure after HIV-1 attachment renders cells more susceptible to subsequent HIV-1 nucleocapsid entry, as occurs in syntenin-depleted cells.

Higher HIV-1-mediated cell fusion and viral entry in syntenin-1-depleted target T-cells also correlates with increased PIP$_2$ production at Env-mediated cell–cell contacts. PIP$_2$ is an important second messenger that targets proteins to the plasma membrane and regulates many actin-related proteins, such as ERMs, filamin, cofilin, and α-actinin (Saarikangas et al., 2010). Generation of PIP$_2$ triggers local actin cytoskeleton changes by activating ERMs (Nakamura et al., 1999), recruiting cofilin (van Rheenen et al., 2009), and inhibiting α-actinin bundling activity (Corgan et al., 2004). Overall, these pathways might favor HIV-1 membrane fusion by facilitating receptor clustering and subsequent actin clearance (Liu et al., 2009). Remarkably, although PIP2-associated cofilin is inactive in tumor cells, cofilin activation in leukocytes is mainly regulated by dephosphorylation (van Rheenen et al., 2009), which is in fact induced upon HIV-1 contact through CXCR4-derived signaling (Yoder et al., 2008). Thus PIP2 might switch the association of membrane receptors and their cytosolic partners (Zimmermann et al., 2005; Saarikangas et al., 2010), modulating the links between transmembrane proteins and the subcortical actin cytoskeleton, and subsequently altering the association of proteins belonging to the same signaling pathway. In this

**FIGURE 5:** Syntenin-1 specifically inhibits viral entry and coclusters with fluorescent VLPs at the plasma membrane. (A) Effect of syntenin-1 silencing on HIV-1 attachment (4°C) and entry (37–4°C) into target T-cells. Data present the fold induction relative to control cells (mean ± SD of three experiments performed in triplicate; p = 0.016). (B) HIV-1 entry in syntenin-silenced cells measured by infection with one-cycle viral particles carrying a luciferase reporter gene. Data present the fold induction of luciferase-HIV-1-envelope virus relative to control cells and normalized to values obtained with luciferase-VSV-envelope virus (mean ± SD from four independent experiments performed in triplicate; p = 0.038). (C) Confocal images showing the recruitment of endogenous syntenin-1 toward attached VLPs (Gag-GFP) at 4°C. Images are projections of the planes in which each individual VLP is observed. Scale bar: 5 μm. Syntenin-1 staining is shown in pseudocolor intensity-coding format next to the confocal image. The small panels are zoomed views of VLP contacts from pseudocolor syntenin-1 and merged immunostaining images (×3). Note that the maximum intensity of syntenin-1 staining (white on the pseudocolor scale images) overlaps the VLP attachment site.

(D) Confocal images showing endogenous syntenin-1 distribution in target T-cells with internalized VLPs (37°C). Two VLPs are shown: one with low syntenin-1 clustering and another with no clustering. Scale bar: 5 μm. Small panels as in (C). (E) Confocal images showing endogenous syntenin-1 distribution in target T-cells forming cell–cell contacts with cells infected with VLPs. Contacts are productive sites for viral transfer and also recruit syntenin-1. Scale bar: 5 μm. Small panels as in (C). (F) Quantification of the number of VLPs that cluster syntenin-1 at the surface of target T-cells (4°C, attached particles), in the cytoplasm (37°C, intracellular particles) or at cell–cell contacts (37°C). Results are from at least three independent experiments and more than 100 VLPs were quantified for each condition.
FIGURE 6: Actin polymerization is reduced in syntenin-1–depleted T-cells. (A) J77 T-cells were incubated with fluorescent VLPs (Gag-GFP) for 30 min, fixed, and stained for syntenin-1 and F-actin. The panels show projections of selected confocal planes in which a VLP was observed, with corresponding pseudocolor images below. Scale bar: 5 μm. The zoomed image shows the particle site (boxed region in merged image) together with orthogonal sections. Quantification of VLPs that colocalized with F-actin in cells silenced or not for syntenin-1 is shown at the right. No significant difference was observed. (B) Time profile of F-actin content upon contact with HIV-1 particles, measured by flow cytometry in control or syntenin-depleted cells. Data are means ± SD of four independent experiments. (C) F-actin accumulation at
regard, in a preliminary attempt to assess syntenin-1 partners, we have observed its association with ERMs and PI4PSK-1 (Gordon-Alonso et al., unpublished data). One possibility is that syntenin-1 may be able to prevent ERMs from fulfilling their role of in facilitating HIV-1 entry but without affecting CD4/CXCR4 clustering. A second option is that syntenin-1 is limiting PI4PSK-1 PIP2 production, and therefore, inhibiting HIV-1 entry (Barrero-Villar et al., 2008). The complexity of syntenin-1 relationships deserves further investigation. Alternatively, depletion of syntenin-1 might simply allow a higher pool of free PIP2 to accumulate at the plasma membrane after HIV-1 attachment, where it could mediate the recruitment of proteins, such as, ERMs, that facilitate HIV entry (Barrero-Villar et al., 2008, 2009).

The fact that syntenin-1 clustering around the virus-like particle vanishes after viral entry is consistent with participation of syntenin-1 at the plasma membrane during HIV-1 entry, probably as a consequence of its association with CD4 or its recruitment through PIP2. We therefore propose a dynamic model in which syntenin-1 is first recruited to the viral attachment site through CD4 and/or PIP2. At this location, syntenin-1 would trigger F-actin polymerization via the adaptor protein M-Rip during T cell chemotaxis (Sala-Valdes et al., 2012). This is consistent with the fact that Rac is the Rho GTPase specifically activated during HIV-1 entry (Pontow et al., 2004; Harmon et al., 2010). Because syntenin-1 regulates Rac-triggered F-actin polymerization through the adaptor protein M-Rip during T cell chemotaxis (Sala-Valdes et al., 2012), it is conceivable that this or a similar signaling axis could be involved during HIV-1 entry. Indeed, syntenin-1 Tyr-4 is essential for Rac-mediated T-cell chemotaxis and superantigen presentation (Sala-Valdes et al., 2012) and seems also to be crucial for syntenin-mediated inhibition of HIV infection. Subsequently, F-actin must be locally depolymerized to facilitate the nucleocapsid entry (Yoder et al., 2008; Vasiliev-Shamis et al., 2009), which is favored by F-actin depolymerization (Sala-Valdes et al., 2010). Because PIP2 induction in the absence of syntenin-1. In this context, PIP2 induction in the absence of syntenin-1 might be the signal that shuts down local F-actin polymerization while activating F-actin clearance.

In summary, our results have unveiled a critical regulatory role for syntenin-1 in controlling HIV-1 entry into target T-cells through altering actin polymerization, most likely in a PIP2-regulated manner.

**MATERIALS AND METHODS**

**Cell lines and reagents**

The Jurkat-derived human T-cell line J77 VJ8* J77cl20 was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Cambrex Bioscience, Charles City, IA). The CEM-T4 human T-lymphoblast–like cell line (courtesy of Paul Clapman), and the HxBc2 Jurkat-derived T-cell line (courtesy of Joseph Sodrowski) were obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program and were cultured according to NIH instructions. The chronically infected MT4-NL4.3-GFP cell line (MT4-HIV-GFP) was generated by Nuria Izquierdo-Useros at the laboratory of Martinez-Picado (IrsíCaixa, Barcelona, Spain).

The biotinylated monoclonal anti-CXCR4 antibody was from BD PharMingen. Mouse monoclonal anti–α-tubulin was from Sigma-Aldrich (Saint Louis, MO). Anti-CD4 monoclonal antibodies used were HP2/6 (Gordon-Alonso et al., 2006), OKT4 (ebioscience), RPA-T4 (eBiosciences, San Diego, CA), CD4v4–FITC (BD PharMingen, Franklin Lakes, NJ), and MEM 241 (Abcam, Cambridge, UK). Rabbit polyclonal anti–syntenin-1 was from Synaptic Systems (Göttingen, Germany). The anti-CD45 mAb was clone D3/9, previously described (Gordon-Alonso et al., 2006). Anti-PIP2 mAb was from Santa Cruz Biotechnology (clone 2C11; Santa Cruz, CA). Anti-GFP antibodies (pAb for IP and mAb for WB) were from Living colors (Clontech, Mountain View, CA). HRP-conjugated secondary antibodies were from Pierce (Rockford, IL).

The intracellular fluorescent trackers CMAC, Calcein-AM, and CM-TMR and all fluorochrome-conjugated secondary antibodies and phalloidins were from Molecular Probes (Camarillo, CA). The HIV-1-specific fusion inhibitor T20 (Enfuvirtide) was from Roche Diagnostics.

**Cell transfection, DNA and siRNA**

J77 cells (2 × 10⁶) were electroporated in cold Optimem (Life Technologies-BRL, Camarillo, CA) with DNA (20 mg) or with siRNA (1.25 mM) using a Bio-Rad GenePulser II electroporator (240 V, 950°F). Fluorescent protein expression and siRNA knockdown were tested respectively by flow cytometry (24 h) and Western blot analysis (48 h).

The syntenin-1 GFP fusion proteins Psrx-HAGFP-syntenin-1 and syntenin-1-Y1-GFP have been described previously (Sala-Valdes et al., 2012). PIP2-enriched plasma membranes were monitored during Env-mediated membrane fusion or VLP attachment by transfecting cells with the biosensor PLC8-PH-GFP (Varnai and Balla, 1998). GST-syntenin-1-CA and GST-syntenin-1-Myc were kindly donated by F. Lozano (Hospital Clinic, Barcelona, Spain).

Negative control siRNA was from Eurogentec (Serain, Belgium) and the specific siRNAs against syntenin-1 were from Thermo Scientific Dharmacon (oligonucleotides J-008270-08 and J-008270-06).

**Preparation and production of HIV-1 and virus attachment and entry assay**

Preparation of HIV-1 NL4.3 and measurement of viral replication were as previously described (Valenzuela-Fernandez et al., 2005). Gag-GFP and Gag-Cherry fluorescent VLPs were produced as previously described (Izquierdo-Useros et al., 2009) by cotransfecting 293T cells with the HIV Gag-eGFP/Cherry plasmids and the envelope plasmid pHXB2.
For p24 production, T-cells were infected with 100 ng of HIV-1 NL4.3 per million cells for 2 h at 37°C (which is equivalent to 1–2Multiplicity of infection or viral particles per cell), and then extensively washed with medium to remove nonattached viral particles. Infected T-cells were kept in culture for 3 d, at which time supernatants were harvested and p24 concentration measured by ELISA (Innotev HIV-1 antigen mAb; Innogenetic, Ghent, Belgium).

For HIV attachment and entry measurements, T-cells were infected with 20 ng of HIV-1 NL4.3 per million cells for 2 h at 4°C (attachment) or 37°C (attachment + entry), and then extensively washed to remove viral input and lysed with RIPA buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). p24 was measured by ELISA (Innotev HIV-1 antigen mAb; Innogenetic).

**Luciferase virus assay**

Replication-deficient luciferase-HIV-1 particles were kindly provided by Suryaram Gummuluru (Boston University, Boston, MA). Replication-deficient viral particles were produced by cotransflecting 293T cells with the luciferase-expressing reporter virus, HIV/Δ nef/Δ env/Δ luc*, which contains the luciferase gene inserted into the nef open reading frame and does not express env glycoprotein (Yamashita and Emerman, 2004), and a CXCR4 tropic (Lai) env glycoprotein or non–Tropic VSV glycoprotein. Virus stocks were generated by PolyFect transient cotransfection of HEK293T cells (Gummuluru et al., 2002). Two days after transfection, cell-free virus-containing supernatants were clarified of cell debris and concentrated by centrifugation (16,000 × g, 1 h at 4°C) and stored at −80°C until required. HIV-1 virus preparations were titrated by p24-ELISA. T-cells were infected with 200 ng of luciferase-based virus with X4-tropic HIV-1 envelope or VSV envelope per million cells for 2 h. Cells were washed extensively and luciferase activity was measured at 48 h after infection with a luciferase assay kit (Promega Corporation, Madison, WI) on a 1450 Microbetta Luminescence Counter (Wallac, Trilux).

**Env-induced cell fusion assay**

A dual-fluorescence cell-fusion assay was performed as described previously (Gordon-Alonso et al., 2006). Briefly, CMTMR-loaded Env* Jurkat-Hxb2 cells were mixed with calcine-AM–loaded parental or transfected CEM-T4 cells. Fluorescence events were detected 14 h later by flow cytometry. Fusion was quantified as the ratio of double-positive events to single-positive target cells.

**Immunoprecipitation**

T lymphoblasts and CEM T-cells were lysed in 1% NP-40, 1 mM MgCl2, 1 mM CaCl2 in Tris-buffered saline (TBS) supplemented with protease and phosphatase inhibitor cocktails. J77 T-cells (4 × 107) were lysed in 1% NP-40 in TBS supplemented with a protease inhibitor cocktail. Lysates were centrifuged at 11,000 rpm for 10 min at 4°C, and supernatants were mixed with reducing Laemmli buffer and boiled for 5 min. Lysates were separated by SDS–PAGE and immunoblotted with specific antibodies. Protein bands were analyzed using the LAS-1000 CCD system and Image Gauge 3.4 software (Fuji Photo Film, Tokyo, Japan).

**Flow cytometry**

Cells were incubated with TBS 5% BSA for 30 min at 4°C and then with primary monoclonal antibody (30 min at 4°C). After being washed, cells were incubated with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody and analyzed in a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, N.J). Data were analyzed with Cell-Quest Pro (Becton Dickinson).

**F-actin quantification**

Cells were stimulated with HIV NL4.3 free virions (100 ng/million cells), fixed with 4% formaldehyde at different time points, permeabilized with TBS 0.5% Triton X-100 (5 min), and stained with Phalloidin–Alexa647 (Molecular Probes, Camarillo, CA). Mean fluorescence intensity of F-actin staining was analyzed on a FACScalibur flow cytometer (BD Biosciences, Franklin Lakes, N.J).

**Statistical analysis**

Statistical significance was calculated using Student’s t test or the parametric one-way analysis of variance with Bonferroni’s post hoc multiple-comparison test. Significant differences are labeled (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

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