Human Discs Large Is a New Negative Regulator of Human Immunodeficiency Virus-1 Infectivity

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Human immunodeficiency virus (HIV)-1 replication is positively or negatively regulated through multiple interactions with host cell proteins. We report here that human Discs Large (Dlg1), a scaffold protein recruited beneath the plasma membrane and involved in the assembly of multiprotein complexes, restricts HIV-1 infectivity. The endogenous Dlg1 and HIV-1 Gag polyprotein spontaneously interact in HIV-1-chronically infected T cells. Depleting endogenous Dlg1 in either adherent cells or T cells does not affect Gag maturation, production, or release, but it enhances the infectivity of progeny viruses five- to sixfold. Conversely, overexpression of Dlg1 reduces virus infectivity by ~80%. Higher virus infectivity upon Dlg1 depletion correlates with increased Env content in cells and virions, whereas the amount of virus-associated Gag or genomic RNA remains identical. Dlg1 knockdown is also associated with the redistribution and colocalization of Gag and Env toward CD63 and CD82 positive vesicle-like structures, including structures that seem to still be connected to the plasma membrane. This study identifies both a new negative regulator that targets the very late steps of the HIV-1 life cycle, and an assembly pathway that optimizes HIV-1 infectivity.

INTRODUCTION

The human immunodeficiency virus (HIV)-1 life cycle is a highly dynamic multistep process during which viral components encounter several cell machineries, creating multiple interactions that profoundly influence virus replication. Indeed, in addition to the general cell machineries required for virus expression per se, host proteins that modulate HIV-1 replication either negatively (restriction factors) or positively (cofactors) have been identified within the last few years. Some of these proteins interact with the structural polyprotein Gag that plays a role during both the early and late steps of the life cycle (reviewed in Demirov and Freed, 2004; Morita and Sundquist, 2004; Holmes *et al.*, 2007). Thus, Gag serves as docking site for the restriction factors APOBEC3G and TRIM5 α , which interfere with the early steps of replication (Nisole *et al.*, 2005; Holmes *et al.*, 2007). During the late steps, Gag recruits proteins of the endocytic pathway, notably proteins of the ESCRT com-

plexes that function as cofactors for virus budding (Morita and Sundquist, 2004).

HIV-1 Gag is a 55-kDa precursor (Pr55), which contains the structural domains Matrix MAP17, Capsid CAP24, Nucleocapsid NCp7, and p6 as well as the spacer peptides 1 (SP1) and 2 (SP2) (reviewed in Muriaux *et al.*, 2004). These Gag domains drive viral assembly and budding (reviewed in Demirov and Freed, 2004). The MA domain is involved in Gag anchoring to the internal face of cell membranes by virtue of an N-terminal myristate and a cluster of conserved basic residues (Spearman *et al.*, 1994; Freed *et al.*, 1995). The MA domain also contains endocytic-sorting motifs able to recruit the clathrin adaptor protein (Batonick *et al.*, 2005; Dong *et al.*, 2005; Camus *et al.*, 2007) and governs incorporation of the envelope glycoprotein (Env) into nascent particles (Cosson, 1996; Hourieux *et al.*, 2000; Lopez-Verges *et al.*, 2006). The NC pilots genomic RNA (gRNA) selection and incorporation during Gag assembly, and together with the CA and SP1 domains, contributes to Gag multimerization (Mammano *et al.*, 1994; Krausslich *et al.*, 1995; Berthouet *et al.*, 1997; Zhang *et al.*, 1998; Liang *et al.*, 2003). The p6 domain controls the budding process through the recruitment of proteins of the ESCRT complexes that belong to the budding machinery located in late endosomes/multivesicular bodies (Gottlinger *et al.*, 1991; Garrus *et al.*, 2001; Strack *et al.*, 2003). Depending on cell types and/or other factors, virus budding seems to occur at the plasma membrane or in endosomes (Ono and Freed, 2004; Grigorov *et al.*, 2006). HIV-1 egress seems to be gated through special membrane microdomains, notably endosome-like domains (Booth *et al.*, 2006) and microdomains enriched in proteins of the tetraspanin family,

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such as CD63, CD81 and CD82 (Nydegger *et al.*, 2006; Jolly and Sattentau, 2007).

Understanding retrovirus/host cell interactions has long been challenging and has led to the recent discoveries of essential cofactors and restriction factors. Along this line, we previously reported that the human homologue of *Drosophila* Discs Large protein (Dlg1/hDlg/SAP97) is a binding partner of the HTLV-1 Env glycoprotein that regulates HTLV-1 transmission (Blot *et al.*, 2004). Dlg1 is a cytosolic protein that is recruited beneath the plasma membrane upon cell contacts. There, Dlg1 functions as a scaffolding, anchoring, and adaptor protein, allowing the assembly of multi-protein complexes and their connection to downstream signaling molecules and/or to cytoskeleton-associated molecules (Funke *et al.*, 2005). Dlg1 belongs to the superfamily of membrane associated guanylate kinases (MAGUKs), which are characterized by a similar structural organization forming protein interacting modules (Funke *et al.*, 2005). Thus, Dlg1 possesses three PSD95/DLG/ZO-1 (PDZ) domains that bind the cytoplasmic tail of integral membrane proteins, a Src homology domain type 3 (SH3) domain, believed to recruit cell signaling molecules (Hanada *et al.*, 1997), a variable HOOK domain, and a guanylate kinase (GUK) domain that is catalytically inactive but recruits cytoskeleton-associated proteins (Funke *et al.*, 2005). Dlg1 is expressed in most tissues and has been readily detected in human T cell lines (Xavier *et al.*, 2004), activated primary T cells (Blot *et al.*, 2004), and mouse primary CD4⁺ T cells (Round *et al.*, 2005). During T cell activation, Dlg1 rapidly translocates to the immunological synapse and associates with early players of T cell activation (Xavier *et al.*, 2004; Round *et al.*, 2005).

In the aforementioned study (Blot *et al.*, 2004), we found that Dlg1 binds to the cytoplasmic tail of HTLV-1 Env and that Dlg1 colocalizes with HTLV-1 Env and Gag at the plasma membrane of T lymphocytes. We further obtained evidence that Dlg1 also interacts with HTLV-1 Gag, at least in vitro (unpublished data). Because similar cell host factors have been shown to be recruited by Gag proteins from distinct retrovirus families (Demirov and Freed, 2004), we investigated whether Dlg1 could interact with other retroviral Gag proteins. In this study, we identify Dlg1 as a novel binding protein for HIV-1 Gag that modulates the distribution of the HIV-1 structural proteins and HIV-1 infectivity.

MATERIALS AND METHODS

Cells

293T cells and HeLa-P4.2 cells were maintained in DMEM, and HIV-1 infected or noninfected Jurkat, CEM, MOLT-4, or Molt-NL4-3 CD4⁺ T cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. The CEM-shDlg cell line was obtained by transducing CEM T cells with retroviral particles (generated by Vectalys, Labège, France) encoding both a short hairpin RNA (shRNA) sequence directed to Dlg1 (same sequence as the anti-Dlg small interfering RNA [siRNA]; see below) and the green fluorescent protein (GFP) gene. Transduced cells were cultivated for 7 d, and the GFP-positive population was sorted by flow cytometry.

Antibodies

Anti-Cap24 rabbit and mouse antibodies were obtained from the National Institutes of Health (Bethesda, MD) AIDS Research and Reference Reagent Program, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and anti-lysosomal membrane protein (LAMP) 2 monoclonal antibodies (mAb) were from Santa Cruz Biotechnology (Tebu, France), and the anti-HIV-1 gp120 110-H and anti-gp41 41-A mAbs were from Pasteur Institute (Paris, France). A serum obtained from an HIV-1-infected patient (anti-HIV-1 serum) was also used for Gag precipitation or detection. Dlg1 was precipitated using a rabbit serum raised against the N-terminal domain of Dlg1, a region that is unique to Dlg1 among MAGUK family members. For immunoblots, Dlg1 was detected using either the 2D11 mAb also directed against the N-terminal

domain of Dlg1 (Santa Cruz Biotechnology) or the anti-PDZ mAb (clone 0.T.124; Euromedex, Souffelweyersheim, France). The mAb against CD63 (TS63) and CD82 (γC11) were kindly provided by H. Conjeaud (Institut Cochin, Paris, France).

Plasmids and siRNA

The pHIV-1 LAL2 proviral plasmid was obtained from the AIDS Research and Reference Reagent Program. The pcDNA3-Gag vector used in the glutathione transferase (GST) pull-down assay was obtained by cloning the polymerase chain reaction (PCR)-amplified Gag sequence of the SVC21BH10 isolate into pcDNA3. Stop codons were introduced in the gag gene by PCR-coupled mutagenesis, allowing the production of truncated gag proteins MACASPINC and MACA. The GST proteins coupled to the different Gag domains have been described previously (Douaisi *et al.*, 2004). The HIV-1 Env expressor pMA-243 (ΔGag/Pol LAI provirus) (Schwartz *et al.*, 1994) was obtained from M. Alizon (Paris, France). The plasmid encoding SAP97, the rat homologue of Dlg1 (93% identity with human Dlg1) that contains the I1 and I3 inserts (Godreau *et al.*, 2003) was kindly provided by S. Hatem (INSERM 621, Paris, France). The Dlg1 domains were PCR amplified from the pCDNA3-Dlg1 vector (Lue *et al.*, 1994) and cloned in frame with the GST sequence in the pGEX-2T vector. The control siRNA (Blot *et al.*, 2004) and the siRNA directed to the N-terminal domain specific for Dlg1 (UGAAGU-GAUAGGUCCAGAA, nucleotides 569–587; accession no. NM_004087) were obtained from QIAGEN (Courtaboeuf, France). The same nucleotide sequences were used to produce the control and anti-Dlg1 shRNA plasmid using the lentiviral vector described in Mangeot *et al.* (2004).

Transfection and Infection

293T cells in 10-cm Petri dishes were transfected using the calcium phosphate procedure with 2.5 μg of pHIV-1 LAL2. The total amount of DNA was maintained at 5 μg by using the pSG5M vector. For Dlg1 knockdown in 293T, two rounds of transfection were performed, the first with 5 μl of a 100 μM solution of siRNAs and the second, 24 h later, with both 2.5 μg of pHIV-1 LAL2 and the same amount of siRNAs. MOLT-4 and Jurkat T cells (1.5 × 10⁶ cells/well) were transfected in six-well plates using 6 μl of DMRIE-C reagent (Invitrogen, Cergy-Pontoise, France) mixed with 4 μg of pHIV-1 LAL2 and either 2 μg of a lentiviral vector producing the shRNA sequences or 2 μl of a 100 μM solution of siRNA.

For HIV-1 LAI production, 293T cells were transfected with 5 μg of the pLAI2 plasmid, and supernatants were collected after 48 h of culture and were conserved at –80°C. The amount of virus was measured using enzyme immunoassay (EIA) assay (see below). For cell infection, 10⁷ CD4⁺ CEM T cells were incubated with 200 ng of virus in 1 ml of Complete RPMI medium containing 10 mM HEPES and 2 μg/ml DEAE dextran for 3 h at 37°C, and then they were washed twice in culture medium and kept in culture for 7 d before use.

GST Pull-Down Assay

GST proteins were produced in *Escherichia coli*, immobilized on glutathione-agarose beads (Sigma, Paris, France) and stored at 4°C. Gag or Dlg1 proteins were synthesized in the presence of [³⁵S]methionine and cysteine (ProMix; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) using the TNT quick-coupled transcription/translation system (Promega, Charbonnières-les-Bains, France). Equal amounts of immobilized GST-proteins were incubated with equal amounts of radiolabeled Gag proteins in binding buffer (50 mM HEPES, pH 7.0, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 0.1% NP-40, 50 mM NaF, and Complete protease inhibitors [Roche Applied Science, Meylan, France]) during 4 h at 4°C. After five washes in binding buffer, the bound proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and the radioactive species were quantified using a Storm-850 PhosphorImager (GE Healthcare). For the RNase/DNase assay, Gag proteins produced using the TNT system were preincubated with RNase or a DNase/RNase mix before performing the GST-Dlg1 pull-down assay. Pulled-down Gag proteins were then revealed by Western blot using the mouse anti-p24 antibody as primary antibody.

Protein Extracts, Immunoprecipitation, and Western Blot

Cells were lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.2, 15 mM NaCl, 1% NP-40, 1 μM Na₃VO₄, 1 mM okadaic acid, and Complete protease inhibitors (Roche Applied Science) for 30 min on ice and centrifuged for 20 min at 14,000 rpm. Proteins were precipitated using protein A-conjugated agarose beads preincubated with 2 μg of appropriate antibodies for 30 min on ice before addition of cell lysates for an overnight incubation at 4°C. For control experiments, excess of control serum was used to ensure the detection of any possible unspecific recognition. Immunoprecipitates were washed five times in lysis buffer and boiled in 2× Laemmli buffer. For control of protein expression, 50 μl of cell lysates was mixed with 2× Laemmli buffer and boiled. Purified virions were obtained from filtered culture supernatants by centrifugation through a cushion of 25% sucrose in TNE (100 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA), at 35,000 rpm for 1 h at 4°C in a Beckman SW21 rotor. For the incorporation experiment, viruses were first

centrifuged through a 25–45% sucrose gradient. Virus pellets were lysed in Laemmli buffer, and total viral proteins were analyzed as described above. Gag proteins were detected using a 1/2500 dilution of the anti-HIV human serum or a 1/5000 dilution of the mouse anti-p24, endogenous Dlg1 by using a 1/2000 dilution of the anti-PDZ mAb, HIV-1 Env gp160, or gp120 with a 1/1000 dilution of the 110-H mAb, HIV-1 gp41 with a 1/1000 dilution of the 41A mAb, and GAPDH with a 1/2000 dilution of the mouse anti-GAPDH mAb. All peroxidase-coupled secondary antibodies were from Promega and were used at a 1/5000 dilution. Scanning of Western blot membranes was performed with an EPSON device, and signal densities were calculated using the ImageJ software (W. Rasband, National Institutes of Health).

Quantification of Gag Production and Virus Infectivity

Forty-eight hours after transfection, 5 ml of fresh medium was added, and the cells were cultivated for 24 h. Supernatants were harvested, centrifuged for 5 min at 3000 rpm and filtered through a 0.45- μ m pore size filter. Virus production was assessed by measuring p24 concentration using a commercial antigen capture EIA (INNOTEST HIV antigen mAb; InGen, Chilly Mazarin, France), according to the manufacturer's instructions. The infectivity of viral supernatants was determined using the indicator HeLa-P4 cells that express CD4 and carry the bacterial lacZ gene under the control of the HIV-1 long terminal repeat (LTR). The day before infection, 8×10^3 HeLa-P4 cells were plated in triplicate in 96-well plates. The cells were incubated with volumes of supernatants adjusted to contain 1 ng of CAp24 for 36 h at 37°C, and then they were washed and fixed. β -Galactosidase activity was measured by a colorimetric assay based on cleavage of chlorophenol red- β -D-galactopyranoside (CPRG), as described previously (Dumoncaux *et al.*, 1998).

RNA Isolation and Quantitative PCR

Virions were purified from filtered supernatants by centrifugation through a cushion of 25% sucrose in TNE, at 35,000 rpm for 1 h at 4°C in a SW21 rotor (Beckman Coulter, Fullerton, CA), and RNA was isolated using the RNeasy mini kit (Invitrogen). The efficiency of the reverse transcription (RT)-PCR reactions was normalized by adding Alien QRT-PCR Inhibitor Alert (Stratagene, Amsterdam, The Netherlands) to each sample. For cDNA synthesis, the following reagents were added to equal amounts of RNA: 32 units of Moloney murine leukemia virus-reverse transcriptase (Invitrogen), 0.5 mM dNTP, 5 mM 1,4-dithiothreitol, 4 μ l of first-strand buffer (Invitrogen), 40 units of RNase Out Recombinant Ribonuclease Inhibitor (Invitrogen), and 150 ng of random primers (Invitrogen). The mix was incubated at 37°C for 50 min, and the reaction was stopped by increasing the temperature to 70°C for 15 min.

Quantification of the HIV-1 cDNA copy number was performed by real-time PCR by using previously described HIV-1-specific primers that anneal in the U5 region of the LTR (MH 531: 5'-TGTGTGCCCCGTCTGTGTGT-3') and in the upstream region of *gag* (MH 532: 5'-GAGTCTGCGCTCGAGAGAGC-3') and primers specific for the Alien sequence provided by the manufacturer. U5-*gag* sequences were amplified in duplicate from 1/10 of cDNA solution in reaction mixtures containing 1 $\times$ Light Cycler Fast Start DNA Master SYBR Green (Roche Applied Science), 4 mM MgCl₂ and 300 nM (each) forward and reverse primers in a final volume of 10 μ l. After an initial denaturation step (95°C for 8 min), 50 cycles consisting of 95°C for 10 s, 60°C for 10 s, and 72°C for 6 s were performed. The copy numbers of HIV-1 cDNA was determined in reference to a standard curve prepared by amplification of quantities ranging from 5×10^3 to 5×10^6 copies of cloned DNA with matching sequences.

Confocal Microscopy

Cell stainings were performed 36 h after transfection or 7 d after infection. For intracellular staining, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized in permeabilizing buffer (PBS, 2% bovine serum albumin [BSA], and 0.1% Tween 20) for 20 min at room temperature. All subsequent incubations and washes were performed in permeabilizing buffer. Cells were stained using the following primary antibodies: anti-CAp24 rabbit serum (1/4000) for Gag immunolocalization, anti-gp120 110H mAb (1/1000) for Env, 2D11 mAb (1/20) for Dlg1, and anti-CD63 (1/1000), anti-CD82 (1/1000), or anti-LAMP2 mAb (1/1000) and then with goat anti-mouse or goat anti-rabbit antibodies conjugated to Alexa 488 (green), Alexa 594 (red), or cyanine 5 (far red). For cell surface staining, cells were fixed as described above but not permeabilized, and Env proteins were stained using the anti-gp120 antibody (1/1000) in PBS containing 2% BSA. Cells were mounted in Mowiol (Merk Eurolab, Pitiviers, France) and examined at 20–25°C. Images were obtained with a TCS SP2 AOBS confocal microscope (Leica, Rueil-Malmaison, France) by using a 63 $\times$ objective. Images were analyzed using ImageJ software.

RESULTS

Dlg1 Modulates HIV-1 Particle Infectivity but Not Particle Release

To evaluate the impact of Dlg1 on HIV-1 replication, we studied the consequence of depleting Dlg1 from producer

cells on HIV-1 virion production and infectivity (Figure 1). 293T cells were transfected once with the HIV-1 LAI proviral DNA plasmid and twice with a control or a Dlg1 siRNA, and analyses were carried out 72 h after transfection. Western blot analyses showed that the siRNA directed against Dlg1 efficiently down-regulates endogenous Dlg1 synthesis (Figure 1A, left top). This reduction did not affect Gag production or maturation in cells, and the amount of CAp24 released in supernatants was also unchanged (Figure 1A, middle). We next quantified the effect of Dlg1 knockdown on the production and infectivity of HIV-1 virions released by 293T cells (Figure 1B). Compared with control cells (siCtl), similar amounts of Gag were produced ($100 \pm 16\%$) and released ($134 \pm 25\%$) by Dlg1-depleted cells (siDlg1). Normalized amounts of virions (1 ng of CAp24) were used to infect indicator HeLa-P4.2 cells, and the level of β -galactosidase activity produced as a result of provirus integration was measured after 36 h of culture (Figure 1B). We found that HIV-1 virions produced by Dlg1-depleted 293T cells were 5 times more infectious than those produced from control cells ($528 \pm 117\%$). To evaluate the impact of Dlg1 depletion in a more physiological cell system, similar experiments were performed in T cells (Figure 1C). MOLT-4 T cells were cotransfected with the LAI plasmid and a lentiviral vector producing either an shRNA targeting Dlg1 or a control shRNA. Dlg1 depletion in transfected T cells ($\sim 15\%$ of total cells) was confirmed by confocal microscopy (data not shown). Compared with control cells, similar amounts of CAp24 were found in MOLT-4 T cells expressing the Dlg1 shRNA in either T cell lysate ($106 \pm 8\%$) or supernatant ($105 \pm 16\%$) (Figure 1C). Similarly to the effect found in adherent cells (Figure 1B), Dlg1 knockdown significantly increased the infectivity of viruses produced by T cells ($556 \pm 211\%$, compared with 100% for control virions; Figure 1C).

To further document the role of Dlg1 and to rule out a nonspecific effect of the Dlg1 si/shRNA, the impact of overexpression of Dlg1 on HIV-1 infectivity was examined. The HIV-1 LAI plasmid was transfected into 293T cells in the presence or absence of a plasmid encoding the rat isoform of Dlg1 (SAP97), and the infectivity of equal amounts of virions (1 ng of CAp24) was analyzed 24 and 48 h after transfection (Figure 1D). As expected, transfection of the SAP97 construct led to an increase in the level of total Dlg1 in 293T cells (Figure 1D, inset). Moreover, overexpression of SAP97 inhibits particle infectivity by 70–80%, in a dose-dependent manner (Figure 1D).

Collectively, our findings demonstrate that Dlg1 is a new important regulator of HIV-1 replication that is dispensable for Gag production and virus release but critical in determining particle infectivity.

Endogenous Dlg1 Associates with Gag but Not Env in HIV-1-infected T Cells

The impact of modulating Dlg1 levels in producer cells suggested that Dlg1 could be an interacting partner of HIV-1 structural proteins. We therefore investigated whether endogenous Dlg1 could be coprecipitated with HIV-1 Env or Gag proteins (Figure 2). Proteins from either noninfected or HIV-1-infected CEM T cells were immunoprecipitated with a control preimmune rabbit serum or a rabbit serum directed against the PDZ domains of Dlg1, and the proteins recovered were revealed using an anti-HIV-1 human serum. In the conditions used, this serum essentially detects Gag proteins, as shown by direct analysis of cell extracts from HIV-1-infected T cells (Figure 2A, lane 2). Immunoprecipitation with the anti-Dlg1 rabbit serum allowed the recovery

Figure 1. Dlg1 is a negative regulator of HIV-1 infectivity. (A) 293T cells were transfected with a control plasmid (Mock) or with the HIV LAI plasmid and either the control (siCtl) or anti-Dlg1 (siDlg1) siRNA, and cell lysates and supernatants were collected 72 h after transfection. Total proteins were analyzed by Western blot by using the anti-PDZ mAb for endogenous Dlg1 (top), the anti-HIV-1 human serum for Gag (middle), and the anti-GAPDH mAb for the loading control (bottom). The data are from one representative experiment out of three performed. (B and C) 293T cells (B) or MOLT-4 T cells (C) were transfected with the HIV LAI plasmid and either the control or anti-Dlg1 siRNA, and cell lysates and supernatants were collected 72 h after transfection. The amounts of intracellular (Cells) and extracellular (Virions) Gag proteins were measured using the anti-CA-p24 EIA. For infectivity, the equivalent of 1 ng of p24 was added to P4.2 cells (HeLa-HIV-LTR-lacZ) and β -galactosidase production was assessed by the CPRG method. Virus production and infectivity were normalized to control siRNA conditions (100%). The results are the mean and standard deviations of at least two independent experiments performed in duplicates. (D) 293T cells were transfected with the HIV LAI plasmid and either 0.2 or 0.5 μ g of a plasmid encoding the rat version of Dlg1 (SAP97), and cell supernatants were harvested 24 and 48 h after transfection. The equivalent of 1 ng of p24 was added to P4.2 cells, and infectivity was measured as described above. The data represent the mean of one representative experiment out of two performed. The inset shows the production of total Dlg1 (endogenous + transfected) in cells seen by immunoblot.

of Pr55Gag in HIV-1-infected CEM T cells (lane 4) but not in noninfected CEM (lane 3). In contrast, no specific signal was found when cell extracts were immunoprecipitated with a control rabbit serum (Figure 2A, lanes 5 and 6). Importantly, similar Gag/Dlg1 coprecipitation was detected using another T cell line infected with a different HIV-1 strain (MOLT-NL4.3; Figure 2B).

Whether Dlg1 interacts with HIV-1 Env was next investigated. Total proteins from MOLT-NL4.3 T cells were precipitated with anti-Dlg1 serum and blotted with an anti-gp120 antibody. Neither gp160 nor gp120 were found in the anti-Dlg1 precipitates (Figure 3A, top), whereas Gag was readily detected with anti-Gag antibodies (Figure 3A, bottom). Blotting with an anti-gp41 antibody produced a strong background in this cell system (data not shown). Whether gp41 can associate with Dlg1 was then studied in 293T cells transfected with the HIV-1 Env expressor pMA-243 (Figure 3B). No band corresponding to gp41 was detected in the anti-Dlg1 precipitate despite proper precipitation of Dlg1 (Figure 3B, top) and high levels of gp41 production in transfected cells (cell lysate, Figure 3B, bottom). Hence, Dlg1 does not interact with HIV-1 Env proteins.

Finally, we examined whether Dlg1 is incorporated within progeny virions. Extracellular viral particles produced from HIV-1-transfected 293T cells were ultrapurified by successive centrifugations through a 25–45% sucrose gradient and a 25% sucrose cushion (Figure 3C). No product was detected when virion lysates were blotted with the anti-PDZ antibody, whereas Dlg1 was readily detected in corresponding cell extracts. This indicates that HIV-1 particles do not contain detectable levels of Dlg1.

These analyses show that the endogenous form of Dlg1 associates with Gag, but not Env, in HIV-1-infected T cells.

However, this robust Gag/Dlg1 interaction does not lead to Dlg1 incorporation into HIV-1 particles.

Pr55Gag Directly Interacts with Dlg1 through the NC Domain of Gag

To identify the Gag domain involved in Dlg1 binding, *in vitro* GST pull-down assays were performed using a GST-Dlg1 fusion protein and either full-length or truncated versions of Pr55Gag (Figure 4A, left). Equal amounts of radiolabeled Gag proteins (Figure 4A, right; see input in lanes 1, 4, and 7) were incubated with identical amounts of GST-Dlg1, allowing quantification of the proportion of Gag proteins pulled down by Dlg1. The full-length Pr55Gag was pulled down by beads coupled to GST-Dlg1 but not GST alone, showing the specificity of the assay and more importantly, indicating that Dlg1 and Gag directly interact. Deletion of the SP2-p6 domains caused a 30% (Figure 4A, lane 6) reduction in Gag binding to Dlg1 compared with binding to full-length Gag taken as 100% (lane 3). The interaction was abolished by further deleting the SP1-NC sequences (Figure 4A, lane 9). The interaction of Dlg1 with various GST-Gag fusion proteins was also studied (Figure 4B). Similar amounts of each GST-Gag proteins were used (Figure 4B, bottom). No binding was found with GST (Figure 4B, lane 1), GST-CA (Figure 4B, lane 4), or GST-p6 (Figure 4B, lane 6). In contrast, radiolabeled Dlg1 was pulled down by the GST constructs fused to a Gag- Δ MA protein (Figure 4B, top, lane 3) or to the NC domain alone (Figure 4B, lane 5). Together, these results indicate that the NC domain of Gag is necessary and sufficient for the interaction with Dlg1.

Because NC is primarily an RNA-binding protein, one could postulate that the Dlg1/Gag interaction is mediated by RNA. However, we concluded that Dlg1/Gag interact

Figure 2. Endogenous Dlg1 interacts with Gag in HIV-1-infected T cells. (A) Cell extracts from noninfected (CEM) or HIV-1-infected (CEM-IIIIB) CEM T cells were immunoprecipitated using either a rabbit serum directed to the N-terminal part of Dlg1 (IP Dlg1) or a control rabbit serum (IP Cont). Total (Lysates) or precipitated (IP) proteins were blotted using an anti-PDZ mAb for endogenous Dlg1 (top) or the anti-HIV-1 human serum for Gag (bottom). (B) Cell extracts from Molt T cells chronically infected with the NL4.3 HIV-1 strain (Molt-NL4.3) were precipitated with the control or anti-Dlg1 rabbit serum as described above, and total (Lysates) or precipitated proteins (IP) were blotted using the anti-HIV-1 human serum. Ig design the heavy chains of the antibody used for the immunoprecipitations.

independently of the specific recognition of the gRNA by the NC domain of Gag because the Gag construct used in the GST pull-down experiments (pCDNA3-Gag; Figure 4, A and C) leads to the synthesis of a truncated HIV-1 RNA lacking the encapsidation signal. To examine whether cellular RNAs bound to the NC domain of Gag can play a role in the Dlg1/Gag interaction, we also performed GST-pull-down experiments by using Gag preparations containing or not nucleic acids. Nonradiolabeled Pr55Gag proteins produced *in vitro* in rabbit reticulocyte lysates were pretreated with either RNase or an RNase/DNase mix before incubation with GST-Dlg1 beads. Pull-down Gag proteins were then revealed by Western blot by using an anti-p24 antibody (Figure 4C). The presence of RNase and/or DNase does not modify the amount of Pr55Gag pulled down by Dlg1 (Figure 4C). Moreover, addition of the same amount of RNase or DNase/RNase mix during the transcription/translation assay prevents the synthesis of Pr55Gag, demonstrating the activities of the enzymes (data not shown). These results suggest that binding of Dlg1 to Pr55Gag does not require gRNA and is not mediated by large NC-bound RNA.

Finally, reciprocal GST-pull down assays were performed to identify the Gag binding domains of Dlg1, by using GST proteins fused to each domain of Dlg1 (Figure 4D, left). The same amounts of GST or GST-Dlg1 proteins (Figure 4D, right bottom) were incubated with *in vitro* translated radiolabeled Pr55Gag, and pulled down proteins were revealed by autoradiography (Figure 4D, right top). No Gag products were recovered with the GST control protein (Figure 4D, lane 2) or with the GST-Nter and GST-PDZ fusions (Figure 4D, lanes 4 and 5). In contrast, Pr55Gag was detected in reactions per-

Figure 3. Endogenous Dlg1 does not recruit gp160, gp120, or gp41 Env proteins and is not incorporated within virions. (A) Cell extracts from noninfected (Molt) or HIV-1-infected (Molt NL4-3) Molt T cells were precipitated using control or anti-Dlg1 rabbit serum, and total (Lysates) or precipitated proteins (IP) were blotted using either the anti-gp120 110-H mAb (top) or the anti-HIV-1 human serum for Gag (bottom). (B) Cell extracts from 293T cells transfected with the HIV-1 Env expressor plasmid pMA-243 were precipitated as in A, and total (Lysates) or precipitated (IP) proteins were blotted using either the anti-PDZ mAb for endogenous Dlg1 (top) or the anti-gp41 41A mAb (bottom). (C) 293T cells were transfected with a control plasmid (Mock) or the LAI proviral plasmid and lysates from cells or ultrapurified virions were prepared 24 h after transfection. Proteins were blotted using the anti-PDZ mAb for endogenous Dlg1 (top) or the anti-p24 mAb for Gag (bottom). Ig in A and B design the heavy chains of the antibody used for the immunoprecipitations.

formed using the GST-Cter fusion, containing the Dlg1 SH3, I3, and GUK domains (lane 6). Both the GST-SH3 and GST-I3 fusion proteins pulled down Gag (Figure 4D, lanes 7 and 8), whereas GST-GUK did not (Figure 4D, lane 9). These results indicate that Dlg1 and Gag directly interact through the NC domain of Gag and the SH3/I3 region of Dlg1 and that this interaction does not depend on the presence of nucleic acids.

Dlg1 Knockdown Does Not Influence gRNA Packaging but Enhances the Amount of Env in Producer Cells and Virions

Dlg1/Gag interaction could influence different assembly processes, including the packaging of gRNA and the recruit-

Figure 4. HIV-1 Gag and Dlg1 directly interact in vitro. (A) Left, Gag constructs used in the experiments. Similar amounts (see 10% input) of in vitro translated radiolabeled HIV-1 full-length Pr55Gag or truncated MACASP1NC and MACA proteins were incubated with constant amounts of immobilized GST or GST-Dlg1 proteins (right). Bound Gag proteins were resolved by SDS-PAGE and quantified by autoradiography using a Storm-850 PhosphorImager. Ten percent of the Gag input was also loaded into the gel (10% input). The percentage of binding to GST-Dlg1 normalized to the binding of full-length Gag (100%) are indicated under the autoradiography. (B) Top, result of the pull-down experiment. Bottom, amount of each GST-Gag protein used in the reaction. Similar amounts of in vitro translated radiolabeled Dlg1 were incubated with constant amount of immobilized GST alone or GST fused to Gag or Gag isolated domains as indicated. Bound Dlg1 proteins were resolved by SDS-PAGE and visualized by autoradiography using a Storm-850 PhosphorImager. Ten percent of the Dlg1 input was also loaded into the gel (10% input). (C) In vitro-translated Gag proteins were preincubated with RNase, RNase/DNase mix, or water in binding buffer before addition of GST-Dlg1. Pulled down Gag proteins and proteins from a control translation reaction (Empty) were blotted using the anti-p24 antibody. (D) Left, GST-Dlg1 constructs used in the experiments. Top, result of the pull-down experiment and the right bottom panel shows the amount of each GST-Dlg1 protein used in the reaction. Similar amounts of in vitro translated radiolabeled HIV-1 Pr55Gag were incubated with comparable amounts of immobilized GST, GST-Dlg1 or GST fused to isolated domains of Dlg1, as indicated. Bound Gag proteins were resolved by SDS-PAGE and quantified by autoradiography using a Storm-850 PhosphorImager (top left). Ten percent of the Gag input was also loaded into the gel (10% input).

ment of Env into virions. To discriminate between these two possibilities, viral proteins or gRNA were extracted from equal amounts of particles purified on a 25% sucrose cushion. To quantify gRNA copies, cDNA was first produced from virus-associated RNA using RT-PCR, in the presence of heterologous RNA for normalization (see *Materials and Methods*). The amplified cDNA was subjected to quantitative PCR using specific primers. Nonsignificant variations were found in the number of cDNA copies between particles obtained from control cells and cells treated with the Dlg1 siRNA (Figure 5A), ruling out a major effect of Dlg1 depletion on gRNA packaging.

Total proteins from producer cells and purified viruses were next analyzed by Western blot to evaluate the levels of Env production and incorporation into virions in cells expressing or not expressing Dlg1 (Figure 5, B and C). Dlg1 was produced in cells treated with the control siRNA, whereas it was strongly reduced in siDlg1-treated cells (Figure 5B, top). Analysis of viral proteins in total cell extracts showed that the amount of Env increased upon Dlg1 depletion in cells as well as in purified virions (Figure 5B, middle), whereas the amount of Gag was unaffected (Figure 5B, bottom). The levels of Env or Gag in cells and virions in Dlg1-depleted cells, and control cells were then quantified in four independent experiments in which infectivity was increased approximately fivefold (Figure 5C). A threefold increase in the level of cell-associated Env (gp160/gp120) and virus-associated gp120 (323 ± 117 and $373 \pm 23\%$, respectively) was observed. In contrast, there was no increase in the level of cell-associated or virus-associated Gag (106 ± 21 and $116 \pm 27\%$, respectively).

Finally, an experiment was performed in which Env was expressed independently on Gag using a Δ Gag/Pol LAI plasmid (pMA-243). Higher levels of gp160/gp120 as well as of gp41 were observed upon Dlg1 depletion (Figure 5D). These experiments show that Dlg1 knockdown has no effect on Gag production or gRNA packaging, whereas it enhances the level of Env in cells and in virions, through a Gag-independent mechanism.

Dlg1 Knockdown in T Cells Modulates Subcellular Distribution of Gag

Whether the interaction with Dlg1 modulates the subcellular localization of Gag in T cells was next studied by immunofluorescence laser microscopy (Figure 6). In either HIV-1-transfected MOLT-4 (Figure 6, top) or Jurkat (Figure 6, middle) T cells, Gag and Dlg1 were concentrated at the plasma membrane, where they partially colocalized (siCtl). Dlg1 was readily depleted in cells cotransfected with the LAI plasmid and the Dlg1 siRNA. Dlg1 knockdown was associated with a dramatic change in Gag localization because Gag was no more found as a homogenous stain at the plasma membrane but rather concentrated within seemingly intracellular dispersed dots (siDlg). The proportion of cells in which Gag was found at the plasma membrane was then determined in two independent experiments by counting at least 70 cells. Gag was located at the plasma membrane in approximately two thirds of control cells (75 and 71% for MOLT-4 and Jurkat T cells, respectively) and in only one third of Dlg1-depleted cells (25 and 34%, respectively).

To confirm these results, Gag localization was also examined in CEM T cells transduced previously with a lentivirus

Figure 5. Dlg1 knockdown has not effect on gRNA packaging but increases the amount of Env in cells and virus. (A) Virus-associated RNAs prepared from equal amounts of purified particles were reverse transcribed, and the corresponding cDNA molecules were amplified using HIV-1-specific primers. The results are normalized to the amount of gRNA found in control conditions and are the means and standard deviations of two independent experiments. (B) Virus particles produced from 293T cells transfected with a control plasmid (Mock) or with the LAI plasmid and either control (siCtl) or anti-Dlg1 (siDlg1) siRNA were purified from cell supernatants by centrifugation on a 25% sucrose cushion. Total cell proteins (Cells) and virus-associated proteins (Virions) were blotted using the anti-PDZ mAb for endogenous Dlg1 (top), the 110-H mAb for Env (middle), and the anti-HIV-1 human serum for Gag (bottom). The results are from one representative experiment out of four performed. (C) Quantification of the Gag and Env protein levels of experiments presented in B. Relative virus infectivity measured in the same experiments is presented in the right histogram. The results are normalized to siRNA control conditions (100%) and are the means and standard deviations of four independent experiments. (D) 293T cells were transfected with a control plasmid (Mock) or with the HIV-1 Env expressor pMA-243 and either the control (siCtl) or anti-Dlg1 (siDlg1) siRNA. Total cell proteins were blotted with the anti-PDZ mAb for Dlg1, the 110-H mAb for gp160 and gp120, the 41A mAb for gp41, and the anti-GAPDH mAb for the loading control, as indicated.

encoding both a shRNA sequence directed to Dlg1 and GFP (CEM-shDlg T cells; see *Materials and Methods*). The CEM-shDlg cells and the parental CEM T cells were infected by HIV-1 LAI, and immunolabeling for confocal analysis was performed 7 d after infection (Figure 6, bottom). In parental CEM T cells, Gag and Dlg1 appear as intense polarized and homogenous stains at the plasma membrane and partially colocalized. As expected, the expression of endogenous Dlg1 was strongly reduced in CEM-shDlg cells compared with control cells. As seen in MOLT-4 and Jurkat T cells, Gag was no longer found evenly distributed at the plasma membrane in CEM-shDlg but accumulated in dispersed dots close to the plasma membrane or more internally.

These experiments show that the absence of Dlg1 leads to the redistribution of Gag to dispersed punctuate structures and that this effect is restricted neither to a particular T cell line nor to a particular mode of HIV-1 production.

Figure 6. Dlg1 knockdown in T cells modulates the distribution of Gag. (A) MOLT-4 or Jurkat T cells were transfected with the pLAI plasmid and with either the control (siCtl) or anti-Dlg1 (siDlg1) siRNA. Seventy-two hours after transfection, cells were costained for Dlg1 (green) and Gag (red). Colocalization of green and red signals occurs as white pixels. Images are single slices from median section of cells recorded in confocal microscopy using a 63 $\times$ objective. (B) CEM T cells (Dlg1+/GFP-) and CEM-shDlg (Dlg1-/GFP+) were infected with HIV-1 LAI and cultivated for 7 d. Cells were then costained for Dlg1 (blue) and Gag (red). Colocalization of blue and red signals occurs as mauve pixels. Images are single slices from median section of cells recorded in a confocal microscope using a 63 $\times$ objective.

Dlg1 Knockdown Induces Accumulation of Gag and Env in CD63- and CD82-positive Compartments

Several studies have reported that HIV-1 virions can bud from various locations in T cells, including tetraspanin-enriched microdomains (TERM) (Nydegger *et al.*, 2006) and endosomes (Nydegger *et al.*, 2003; Grigorov *et al.*, 2006). Colocalization experiments were therefore performed between Gag, Env, and some markers of these compartments in HIV-1-infected CEM and CEM-shDlg T cells (Figure 7). In parental CEM T cells, CD63 was located in intracellular compartments as well as at the plasma membrane, and the latter population strongly colocalized with Gag (Figure 7A, top left). This is consistent with recent studies showing that a fraction of CD63 is delocalized to the plasma membrane in HIV-1-infected T cells (Jolly and Sattentau, 2007). CD82 was essentially found at the plasma membrane where it strongly colocalized with Gag (Figure 7A, middle left). LAMP2, a

Figure 7. Dlg1 knockdown redistributes Gag and Env in CD63- and CD82-positive dispersed compartments. (A) Parental CEM (Dlg⁺/GFP⁻) or CEM-shDlg (Dlg⁻/GFP⁺) T cells were infected with HIV-1 LAI and cultivated for 7 d. The cells were fixed, permeabilized, and costained for Gag (red) and either CD63, CD82, or LAMP2 (blue). (B) In parallel experiments, cells were costained for Env (red) and either CD63, CD82, or LAMP2 (blue). For CEM-shDlg cells, GFP expression is also shown in the merge. Colocalization of blue and red signals occurs as mauve pixels. Images are single slices from median section of cells recorded in a confocal microscope using a 63× objective.

lysosomal marker, was only found inside the cell and did not colocalize with Gag (Figure 7A, bottom left). In CEM-shDlg1 cells, Gag was redistributed into dispersed dots near the plasma membrane and in larger intracellular compartments, containing CD82 or CD63, but not LAMP2 (Figure 7A, right).

In parental CEM T cells, Env occurred as a polarized or homogenous stain at the plasma membrane (Figure 7B, left). As for Gag, Env colocalized with CD82 and with a fraction of CD63 (Figure 7B, middle and top left). In CEM-shDlg T cells, Env became concentrated in puncta close to the plasma membrane and in larger intracellular compartments. These structures were essentially positive for CD82 or CD63, and in some cases, for LAMP2 (Figure 7B, right).

Costaining of Gag and Env showed that Gag colocalized with the fraction of Env present at the plasma membrane in parental CEM T cells and that Gag and Env were redistributed to the same dispersed puncta in CEM-shDlg cells (Figure 8A). The effect of Dlg1 knockdown on Env transport to the plasma membrane was also investigated by performing staining on nonpermeabilized cells. Capping of Env at the cell surface was observed in parental CEM T cells (Figure 8B,

Figure 8. Env colocalizes with Gag in Dlg1-depleted T cells and accumulates in vesicle-like structures at the plasma membrane. (A) Parental CEM (Dlg⁺/GFP⁻) or CEM-shDlg (Dlg⁻/GFP⁺) T cells were infected with HIV-1 LAI and cultivated for 7 d. Cells were then fixed, permeabilized, and costained for Gag (red) and Env (blue). Colocalization of red and blue signals occurs as mauve pixels. (B) In parallel experiments, CEM and CEM-shDlg T cells were fixed but not permeabilized, and cell surface staining of Env was performed (red). Images are single slices from median section of cells recorded in a confocal microscope using a 63× objective.

left). In CEM-shDlg T cells Env proteins were dispersed in plasma membrane dots and/or as large round vesicle-like structures (Figure 8B, right). Counting of 50 cells showed that Env presented homogenous plasma membrane stain in 68% of CEM T cells and in only 23% of CEM-shDlg T cells. We noticed that Env distributions at the plasma membrane were very similar between nonpermeabilized or permeabilized CEM-shDlg cells. Therefore, some of the vesicular structures containing Env identified in permeabilized cells are still connected with the plasma membrane and may therefore correspond to plasma membrane invaginations.

Together, these observations indicate that depleting Dlg1 induces relocation of Gag and Env from polarized and homogenous plasma membrane sites to large and dispersed

puncta containing CD63 and CD82, including plasma membrane-linked structures.

DISCUSSION

This study demonstrates that the scaffold protein Dlg1 negatively regulates HIV-1 particle infectivity and reveals an optimizing mechanism for particle infectivity linked to the redistribution of the HIV-1 structural proteins in particular cell compartments.

Our conclusion that Dlg1 is a new binding partner of HIV-1 Gag is supported by data obtained both *in vitro* and in cells. Dlg1 directly interacts with Gag *in vitro* and the NC domain of Gag is necessary and sufficient for this interaction. The primary function of the NC domain is to promote encapsidation of the gRNA. However, our data show that Gag and Dlg1 interact *in vitro* independently of the presence of gRNA or cellular nucleic acids and that the level of Dlg1 in producer cells does not modulate gRNA encapsidation. This indicates that the NC domain of Gag also functions as docking site for cell proteins, independently on its RNA-packaging property. Gag binds to the C-terminal SH3 or/and I3 domains of Dlg1, with an apparent higher affinity for the I3 domain. Gag is therefore more likely to bind to the Dlg1 isoform that contains the I3 insert, which is the isoform targeted to the plasma membrane (McLaughlin *et al.*, 2002). This is consistent with our immunofluorescence studies showing that Gag and endogenous Dlg1 colocalize at the plasma membrane of HIV-1-infected T cells.

Importantly, our results also demonstrate that Gag and Dlg1 associate in cells, because complexes formed between Gag and endogenous Dlg1 could be retrieved from HIV-1-infected T cells. Hence, the interaction between endogenous Dlg1 and Gag is strong enough to be detected without overexpressing either protein, and it readily occurs in a major target cell population of HIV-1. The Dlg family contains other members, Dlg2 to Dlg7, which may also interact with Gag in T cells. However Dlg3 (SAP102/neuroendocrine-dlg) and Dlg7 are not expressed in T cells (Makino *et al.*, 1997; Gudmundsson *et al.*, 2007). Moreover, a very similar band pattern was observed in immunoblots performed on T cell lysates when using either the anti-PDZ antibody, which recognizes Dlg1 as well as other proteins of the Dlg family, or the Dlg1 specific antibody (data not shown). Finally, the specific knockdown of Dlg1 is sufficient to affect HIV-1 infectivity. This suggests that Dlg1 is the major Dlg member responsible for HIV-1 modulation in T cells.

From a functional point of view, despite binding to Gag, the level of Dlg1 has no effect on Gag synthesis, maturation, or release. This implies that Dlg1 is not required for Gag assembly into particles, consistent with our finding that Dlg1 is dispensable for gRNA packaging. In line with this conclusion, Dlg1 is not incorporated at detectable levels into progeny virions. However, our data clearly demonstrate that Dlg1 is a potent regulator of HIV-1 infectivity. Depleting Dlg1 enhances particle infectivity five to sixfold, whether particles originate from adherent cells or from T cells. Conversely, virions produced from cells overexpressing Dlg1 are 80% less infectious than control virions. The gain in virion infectivity found upon Dlg1 depletion correlates with higher amounts of Env in both producer cells and virions, providing a direct molecular explanation for the phenomenon observed.

How does Dlg1 level modulate the amount of Env? This effect was not dependent on the presence of Gag, which is consistent with the fact that Dlg1 knockdown has no impact of Gag production in producer cells. Dlg1 depletion could

enhance transcription and/or translation of the Env mRNA. However, this seems unlikely because no change in the level of Gag was detected in the same conditions, indicating that Dlg1 depletion does not grossly influence the production or translation of HIV-1 mRNAs. Direct binding of Dlg1 to Env that would modify Env stability and/or Env trafficking in cells could also be ruled out, because we reported previously that the cytoplasmic tail of the HIV-1 TM (gp41) does not bind to Dlg1 (Blot *et al.*, 2004) and confirm here that neither gp160, gp120 nor gp41 form complexes with Dlg1 in cells. Hence, although Dlg1 binds to HTLV-1 Env, facilitating cell-to-cell viral transmission, it only binds to HIV-1 Gag, inhibiting HIV-1 virion infectivity. Given that the biology of HTLV-1 and HIV-1 is so different, it is not surprising that Dlg1 may not play a similar role in the life cycle of these two retroviruses. Parallel studies of these two viruses using the same techniques is now needed to definitely clarify the role of Dlg1 in cell-to-cell and free virus transmission of HIV-1 or HTLV-1.

Our confocal microscopy analyses could provide another explanation for the effect of Dlg1 knockdown on Env. Indeed, Dlg1 depletion also modifies the subcellular distribution of Env. In parental CEM T cells that produce Dlg1 endogenously, Env occurs as a polarized and homogenous plasma membrane stain. In contrast, in CEM-shDlg T cells lacking Dlg1, Env becomes concentrated in dispersed puncta near the plasma membrane and in larger compartments that extend within cells. Those larger Env-positive compartments are enriched in CD82 and CD63 and in some cases in LAMP2, suggesting that they could correspond to endosomes. Remarkably, some Env-positive dispersed dots superposed or close to the plasma membrane could also be detected when Dlg1-depleted CEM T cells were stained without permeabilization. This suggests that at least some vesicle-like structures containing Env are continuous with the plasma membrane and therefore do not correspond to classical endosomes. These data indicate that Dlg1 depletion does not prevent Env from accessing the cell surface but rather induces Env concentration into plasma membrane invaginations and/or endosomes. It has been reported previously that HIV-1 Env proteins can remain stable for more than 16 h within regulated secretory vesicles of T cells (Miranda *et al.*, 2002). Similarly, Env concentration in the new compartments formed in Dlg1-depleted cells could lead to protein stabilization by diverting Env from degradation.

In addition to its effect on Env, Dlg1 also modulates the distribution of Gag. Indeed, in Dlg1-depleted T cells, Gag and Env colocalize in dispersed dots superposed or adjacent to the plasma membrane and/or in larger compartments and the two proteins colocalize with either CD82 or CD63 in these structures. Hence, absence of Dlg1 induces concentration of Gag and Env in new compartments that seem to correspond to tetraspanin-enriched plasma membrane invaginations and/or endosomes. The striking finding of this study is that these redistributions create a situation that clearly enhances particle infectivity. The identity of the cell sites where HIV-1 virions assemble or bud is still a matter of debate. The prevailing view proposes that budding occurs at the plasma membrane in T cells (Jouvenet *et al.*, 2006), and in endosomes in macrophages (Raposo *et al.*, 2002; Pelchen-Matthews *et al.*, 2003). In T cells, studies have reported that HIV-1 budding occurs at the plasma membrane, in TERM (Nydegger *et al.*, 2006; Jolly and Sattentau, 2007) or in endosome-like domains (Booth *et al.*, 2006). However, other studies reported that HIV-1 virions can also be found in intracellular endosomal compartments in T cells (Nydegger *et al.*, 2003; Grigorov *et al.*, 2006). Moreover, recent reports de-

scribed that in macrophages, HIV-1 particles accumulate in seemingly intracellular vacuolar structures that correspond rather to plasma membrane invaginations or internally sequestered plasma membrane domains. These domains are accessible for extracellular antibodies and are enriched in CD63 and CD82 (Deneka *et al.*, 2007; Welsch *et al.*, 2007). We describe here a situation in which the concentration of Gag and Env into apparently intracellular compartments containing CD63 and CD82, including structures labeled by extracellular antibodies, is associated with enhanced infectivity. We propose therefore that Dlg1 depletion favors the appearance of new cellular sites that could correspond to plasma membrane invaginations similar to those described in macrophages, the validation of this hypothesis requires future immunoelectron microscopy studies. Whatever their exact nature, our present data indicate that these sites function as specialized platforms that optimize the generation of infectious particles. Proteins of the ESCRT complexes have been shown to be distributed throughout the endocytic pathway, including the plasma membrane and tubular-vesicular endosomal membranes, in both T cells and macrophages (Welsch *et al.*, 2006). Hence, we speculate that the concentration of Gag and Env in these structures facilitate not only Gag/Env assembly but also their functional interactions with cellular proteins such as tetraspanins and budding cofactors. Furthermore, our data identify Dlg1 as a host cell factor that regulates the generation of these assembly platforms. Dlg1 is targeted to the inner face of the plasma membrane and is connected to the cytoskeleton (Kim *et al.*, 1997; Wu *et al.*, 1998). Dlg1 knockdown results in the disruption of the actin cytoskeleton in epithelial cells (Firestein and Rongo, 2001) and in impaired antigen-induced actin polymerization in T cells (Round *et al.*, 2005). Remarkably, the formation of invaginations of the plasma membrane could be enhanced by disruption of the actin cytoskeleton (Itoh *et al.*, 2005; Tsujita *et al.*, 2006). These findings along with our data are consistent with a model in which, through disorganization of the actin cytoskeleton, Dlg1 knockdown facilitates the formation of intracellular extensions of the plasma membrane. Conversely, Dlg1 overexpression would either interfere with the formation of plasma membrane invaginations and/or, by virtue of its direct binding to Gag, prevent the targeting of Gag to these structures. This is consistent with the reduced infectivity observed in cells that overexpress Dlg1 and with the fact that Gag interacts with the Dlg1 isoform that is targeted to the plasma membrane. A surprising finding of our study is that Dlg1 modulation impacts virus infectivity but has no effect on the level of virus release. This emphasizes the notion that Gag assembly and release and the process of infectious particle formation are two related but not strictly overlapping phenomena that seem to be regulated by distinct host cell proteins. The exploration of the roles of Dlg1 in plasma membrane dynamics and the distribution of the HIV-1 structural proteins in T cells will constitute therefore a promising approach to elucidate the mechanisms governing the very late steps of the HIV-1 life cycle.

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