Human Dlg protein binds to the envelope glycoproteins of human T-cell leukemia virus type 1 and regulates envelope mediated cell-cell fusion in T lymphocytes

Vincent Blot[1,*], Lélia Delamarre[1], Fabien Perugi[1], Danielle Pham[3], Serge Bénichou[2], Richard Benarous[2], Toshihiko Hanada[4], Athar H. Chishti[4], Marie-Christine Dokhélar[1] and Claudine Pique[5,*]

[1]Département Biologie Cellulaire, and [2]Département Maladies Infectieuses, CNRS UMR 8104 and INSERM U567, Institut Cochin, 22 rue Méchain, 75014 Paris, France
[3]Institut Gustave Roussy, 39 rue Camille Desmoulins, 94805 Villejuif CEDEX, France
[4]Department of Pharmacology and Cancer Center, University of Illinois College of Medicine, Chicago, IL 60607, USA
[5]CNRS UPR 9051, Hôpital St.-Louis, 75475 Paris CEDEX 10, France
*Authors for correspondence (e-mail: vblot@cochin.inserm.fr; pique@chu-stlouis.fr)

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Summary

Human homologue of the *Drosophila* Dlg tumor suppressor (hDlg) is a widely expressed scaffold protein implicated in the organization of multi-protein complexes at cell adhesion sites such as the neuronal synapse. hDlg contains three PDZ domains that mediate its binding to the consensus motifs present at the C-termini of various cell surface proteins, thus inducing their clustering and/or stabilization at the plasma membrane. Using a yeast two-hybrid screen, we identified hDlg as a cellular binding partner of a viral membrane integral protein, the envelope glycoprotein (Env) of human T-cell leukemia virus type 1 (HTLV-1). HTLV-1 is a human retrovirus that infects CD4+ T lymphocytes and is preferentially transmitted via direct contacts between infected and target cells, through a structure referred to as the virological synapse. Here, we demonstrate that hDlg interacts with a classical PDZ domain-binding motif present at the C-terminus of the cytoplasmic domain of HTLV-1 Env and conserved in the related HTLV-2 virus. We further document that, in HTLV-1 infected primary T cells, hDlg and Env are concentrated in restricted areas of the plasma membrane, enriched in molecules involved in T-cell contacts. The presence of Gag proteins responsible for viral assembly and budding in these areas indicated that they constitute platforms for viral assembly and transmission. Finally, a mutant virus unable to bind hDlg exhibited a decreased ability to trigger Env mediated cell fusion between T lymphocytes. We thus propose that hDlg stabilizes HTLV-1 envelope glycoproteins at the virological synapse formed between infected and target cells, hence assisting the cell-to-cell transmission of the virus.

Key words: hDlg, Retrovirus, Envelope, Cell-to-cell transmission, Virological synapse

Introduction

Membrane-associated guanylate kinases (MAGUKs) constitute a family of proteins that play an essential role in the organization of cell-cell adhesion sites such as neurological synapses and tight junctions of epithelial cells (Kim, 1997). In mammals, several subgroups of MAGUKs have been described, including the Disc large (Dlg)-like subgroup composed of PSD95/SAP90, Chapsyn 110/PSD-93, NE-Dlg/SAP102 and hDlg/SAP97 (Fujita and Kurachi, 2000). These proteins share a similar modular organization with three PSD95/Dlg/ZO-1 (PDZ) domains, one Src Homology domain type 3 (SH3) domain, and a C-terminal region homologous to guanylate kinases (GUK) (Gonzalez-Mariscal et al., 2000). PDZ domains mediate clustering of integral membrane proteins that bear a specific signature sequence -E/D-S/T-x-V/L/I at their C-terminus (Harris and Lim, 2001). In addition to interacting with exogenous ligands, the SH3 and GUK domains interact with one another in either an intra or intermolecular fashion (McGee and Bredt, 1999; McGee et al., 2001; Nix et al., 2000; Tavares et al., 2001). Some members of the Dlg-subfamily also contain a positively charged HOOK domain that permits an interaction with proteins of the Ezrin, Radixin, Moesin (ERM) family that are linked to the actin cytoskeleton (Lue et al., 1996). In addition, hDlg contains a N-terminal proline-rich region implicated in self association and binding to proteins involved in signal transduction pathways (Hanada et al., 1997; Tezuka et al., 1999). Through this multitude of protein interactions, MAGUKs act as scaffolding proteins mediating clustering and retention of various macromolecular complexes at the plasma membrane. For example, the PSD95 protein, which is enriched at the postsynaptic density (PSD) in neurons, clusters NMDA receptors and Shaker K+ channels (Mori et al., 1998; Steigerwald et al., 2000) together with signaling proteins, such as the neuronal nitric oxide synthase (Christopherson et al., 1999) and the Fyn tyrosine kinase (Tezuka et al., 1999). By direct or indirect interactions, PSD95 is believed to link proteins

of the PSD to the cytoskeleton (Hirao et al., 2000; Naisbitt et al., 2000) thus preventing their diffusion across the plasma membrane.

hDlg and its rat homologue SAP97 are found in a wide range of cell types. In adherent cells, such as neurons or epithelial cells, it is localized at cell-cell adhesion sites, tightly associated with the cortical cytoskeleton (DeMarco and Strehler, 2001; Laprise et al., 2003; Lue et al., 1994; Reuver and Garner, 1998; Wu et al., 1998). Several domains of hDlg have been shown to have an important role for its intracellular localization, particularly the amino-terminal and GUK domains that interact with microtubule-associated proteins and molecular motors (Hanada et al., 2000; Wu et al., 2002). In resting T-lymphocytes, hDlg exhibits mainly a diffuse distribution in the cytoplasm. However, upon experimental crosslinking of CD2 adhesion molecules that mimics cell-cell contact, hDlg is recruited to the plasma membrane particularly at the crosslinked sites (Hanada et al., 2000). This observation suggests that, similarly to its role in the organization of cell-cell junctions in adherent cells, hDlg could also participate in the establishment of cell-cell contacts in T lymphocytes.

We are interested in studying the transmission of the human T-cell leukemia virus type 1 (HTLV-1), a retrovirus that infects T lymphocytes. Retroviruses are composed of a core that results from the assembly of mature Gag proteins and the viral RNA, surrounded by a lipid bilayer in which the envelope glycoproteins (Env) are anchored. The polyprotein precursor of Gag proteins is responsible for assembly and budding of the virions from infected cells, whereas the envelope glycoproteins mediate virus entry into target cells. Env are composed of the non-covalent association of a surface subunit (SU), involved in receptor(s) recognition and binding, and a transmembrane subunit (TM) that ensures the membrane anchorage of the SU/TM complex (Delamarre et al., 1996; Paine et al., 1994; Pique et al., 1992). Importantly, the TM bears the fusion activity that, by mediating viral and cell membranes mixing, allows virus core entry into target cells. In vitro, this phenomenon also leads to the formation of multinucleated cells called syncytia (Pique et al., 1992).

Unlike other retroviruses, HTLV-1 is almost exclusively transmitted via direct cell-to-cell contacts, the viral particles being non infectious in vivo (Manns et al., 1999). After the establishment of a stable interaction between an infected and a target cell, Gag and Env proteins together with the viral RNA become concentrated at the precise sites of cell contacts and are subsequently transmitted to the target cell (Igakura et al., 2003). These specialized cell contact areas, through which HTLV-1 transmission occurs, were called the virological synapse (Dustin, 2003; Igakura et al., 2003), by analogy to the immunological synapse established between immune cells during antigen recognition (Bromley et al., 2001).

We have previously shown that the cytoplasmic domain (CD) of HTLV-1 envelope transmembrane glycoprotein is implicated in the cell-to-cell transmission of the virus, and postulated that this effect could be mediated through interactions with cytosolic partners (Delamarre et al., 1999). In the present study, we used this domain as bait in a yeast two-hybrid screen against a peripheral blood lymphocyte (PBL) cDNA library and isolated hDlg as a cellular binding partner of the HTLV-1 Env-CD. We demonstrated that hDlg binds to and partially co-localize with native envelope glycoproteins expressed by chronically infected T cells. Moreover, mutant virus expressing envelope proteins unable to recruit hDlg showed a highly reduced ability to trigger Env-mediated cell-to-cell fusion between T lymphocytes. We propose that hDlg stabilizes HTLV-1 envelope glycoproteins complexes at the virological synapse, thus improving envelope/receptor interactions and subsequent virus cell-to-cell spread.

Materials and Methods

Yeast two-hybrid screen and mutagenesis

A cDNA encoding the 22 amino-acids of the HTLV-1 transmembrane glycoprotein cytoplasmic domain (CD) was subcloned in frame with the sequence encoding the DNA-binding domain of LexA into the pBTM116 vector to give the pBTM116 Env-CD plasmid. The PBL cDNA library was constructed in frame with the cDNA of the Gal4 activating domain in the pGAD1318 vector as described (Peytavi et al., 1999). cDNAs encoding either the full length hDlg protein, or the region containing the three PDZ domains were subcloned from the pCDNA3-hDlg vector (Lue et al., 1994) in the pGAD1318 vector. The yeast two-hybrid screen and the yeast two-hybrid assays were performed as described elsewhere (Benichou et al., 1994; Margottin et al., 1998).

Deletion of the sequence encoding the entire PDZ-binding motif (ΔESSL) was performed using the pBTM116 Env-CD plasmid by polymerase chain reaction with the QuikChange Site-Ditected Mutagenesis kit from Stratagene (Ozyme, France). Other CDs with point mutations were isolated from the corresponding CMV-Env-LTR expressors (Delamarre et al., 1999) and were directly subcloned in the pBTM116 plasmid.

Cell lines and antibodies

Jurkat T-cell line was cultured in RPMI medium containing 10% fetal calf serum (FCS) and 2 mM L-glutamine (Life Technologies, France). The CIB cells are HTLV-1-infected activated primary T cells obtained from patient CIB who had inflammatory disorders. They were obtained from CD4+ T cells purified by positive selection after stimulation with phytohemagglutinin (PHA-M, 1 μg/ml, Sigma, France) and cultured for less than 2 months in RPMI medium containing 10% FCS plus 50 U/ml interleukin 2, as described (Pique et al., 2000). Due to short term culture, these cells retain the phenotype and morphology of activated primary T lymphocytes (Wucherpfennig et al., 1992).

Anti-hDlg 2D11 monoclonal antibody (mAb) was used either as ascites fluid (Nix et al., 2000) for western blotting or as purified antibody (Santa Cruz, Tebu, France) for immunofluorescence analysis. The mAb K28/86.2 raised against the PDZ domains of PSD95 recognizes several MAGUKs including hDlg and was purchased from Upstate Biotechnology (Euromedex, France). HTLV-1 envelope glycoproteins at the cell surface were visualized using sera from HTLV-1-infected patients, and Gag proteins were detected using the p19 mAb as described elsewhere (Le Blanc et al., 2002). Anti-CD4 mAb and FITC-conjugated anti-CD25 mAb were obtained from Pharmingen (Becton Dickinson, France), anti-CD2 mAb was from Immunotech (Coulter, France), and the rabbit purified immunoglobulins directed to $p56^{lck}$ were from Santa Cruz (Tebu, France). The biotin-conjugated Cholera toxin was purchased from Sigma (France). All conjugated secondary antibodies were from Jackson Immunoresearch (Interchim, France), and the DTAF-conjugated streptavidin was from Immunotech (Coulter, France).

GST fusion proteins

cDNA encoding the HTLV-1 envelope glycoprotein CD amplified from the CMV-Env-LTR vector (Delamarre et al., 1997) and the full-

length hDlg cDNA amplified from the pCDNA3-hDlg plasmid (Lue et al., 1994) were subcloned in frame with the GST cDNA in the pGEX-3X vector (Amersham Pharmacia Biotech, France). GST, GST-CD, and GST-hDlg fusion proteins were produced in *E. coli*, immobilized on glutathion-sepharose beads (Sigma, France) and stored at –80°C until further utilization.

Precipitation of hDlg from T-cell cytosolic extracts

Jurkat cells (50×10^6 cells per assay) were washed twice with the cytosol buffer (phosphate buffered saline supplemented with 0.7 mM $CaCl_2$, 0.25 mM $MgSO_4$, 2.5 mM $KC_2H_3O_2$ and 1 mM phenylmethylsulfonyl fluoride), and resuspended in 700 µl of the same buffer. Cells were then broken by several freeze/thaw cycles (liquid nitrogen/37°C), and five passages through a 21-gauge syringe. After centrifugation for 30 min at 20,000 ***g*** at 4°C, the supernatant was precleared on GST beads for 90 minutes at room temperature under constant agitation. Precleared cytosol fractions were incubated with GST or GST-CD beads at 4°C overnight, and beads were washed five times in PBS. Proteins were eluted from the beads by boiling for five minutes in the loading electrophoresis buffer. Eluted proteins were separated on SDS-7.5% PAGE, transferred onto PVDF membranes, and subjected to western blotting using either the anti-hDlg 2D11 mAb (ascite fluid, 1/10,000 dilution) or the anti-PSD95 K28/86.2 mAb (1/500) for detection of hDlg and other Dlg-like MAGUKs, followed by peroxidase-conjugated goat anti-mouse secondary antibody. Binding was detected using ECL (Amersham Pharmacia Biotech, France). Coomassie-blue staining normalized the amount of GST beads used in each assay.

Precipitation of HTLV-1 Env from CIB cell lysate

HTLV-1 infected CIB T cells (3×10^6 cells per ml) were incubated overnight in culture medium supplemented with 200 µCi/ml [^{35}S]Met/Cys protein labelling mixture (NEN, Perkin Elmer, France). Cells were washed twice in PBS, and lysed in lysis buffer (Tris-HCl, 20 mM, pH 8, NaCl 120 mM, EGTA 0.2 mM, NP40 0.5%), supplemented with protease inhibitors (Complete, Roche Diagnostic, France) on ice for 30 minutes. After centrifugation for 30 minutes at 20,000 ***g*** at 4°C, the supernatants were incubated with GST or GST-hDlg beads overnight at 4°C under constant agitation. Beads were then washed in lysis buffer, and precipitated proteins were eluted by 5 minutes boiling in the loading buffer. Radiolabelled proteins were separated by SDS-13% PAGE. Control immunoprecipitation of HTLV-1 proteins was performed using 3 µl of sera from HTLV-1-infected patients immobilized on protein G-sepharose beads as described elsewhere (Pique et al., 1992).

Co-localization studies

HTLV-1-infected CIB T cells that grow in culture as tight clusters were mechanically dissociated before being plated onto poly-L-Lysine coated glass slides. Cells were then fixed in 4% paraformaldehyde-PBS for 20 minutes and quenched in 100 mM glycine-PBS for 20 minutes. Non-permeabilized cells were stained for HTLV-1 envelope proteins and cell surface antigens with sera from HTLV-1 infected donors (1/100 in PBS), and either anti-CD2, anti-CD4, or FITC-conjugated anti-CD25, followed by Texas red-conjugated anti-human secondary antibodies (1/300 in PBS) and FITC-conjugated anti-mouse when necessary (1/300 in PBS). Staining of lipid rafts was performed, together with the staining of HTLV-1 Env, using biotin-conjugated cholera toxin (5 µg/ml in PBS) on fixed cells, and visualized using FITC-conjugated streptavidin (1/1600 dilution).

After surface HTLV-1 Env labelling, the cells were permeabilized in permeabilizing buffer (0.05% Saponin, 0.2% bovine serum albumin in PBS) for 40 minutes and stained in permeabilizing buffer for either hDlg, Gag or p56^{lck} using purified 2D11 mAb directed against hDlg (1/40), anti-Gag p19 mAb (1/600) or anti-Lck (1/50) respectively, followed by the appropriate FITC-conjugated secondary antibodies (1/300). After washing, slides were mounted with Mowiol (Calbiochem, Merk Eurolab, France) and examined under a confocal microscope (Model MRC-1024, BioRad, France or Leica TCS SP2, Leica, France). Images were acquired using either the Lasersharp Acquisition software or the Leica Confocal Software and processed using Adobe Photoshop.

Cell-to-cell fusion assay

Jurkat T cells were transfected using electroporation method with either wild-type proviral clone XMT (Derse et al., 1997), or mutated constructs in which the serine 486 of the ES^{486}SL PDZ-binding site in the envelope CD has been changed to a leucine (XMT-Env-S486L), or in which a premature stop codon was introduced in the *env* gene (XMT-delta Env) (Delamarre et al., 1997). To do so, Jurkat T cells were adjusted to 10^7 cells/ml in transfection buffer (RPMI+20% FCS). After 15 minutes at room temperature in the presence of 10 µg of the appropriate XMT proviral vector, together with 3 µg of the HTLV-1 Tax encoding plasmid CMV-Env-ΔPvuII and 20 µg of pcDNA3 empty plasmid, 500 µl of the cell suspension was subjected to an electric pulse (320V, 960 µFa) in a BioRad electroporator and re-suspended in 5 ml of culture medium.

Thirty-six hours after transfection, cells were counted and seeded onto glass slides at the equal density of 10^7 cells/ml to favor cell-to-cell contacts. After 30 or 90 minutes of incubation at 37°C, cells were washed and fixed using 4% paraformaldehyde-PBS, and Env and Gag proteins were detected by immunofluorescence techniques and confocal analysis as described above.

Env mediated cell fusion ability of wild-type versus mutated proviruses was then estimated by counting under a fluorescence microscope the number of syncytial cells, i.e. cells containing more than one nucleus, among all the viral Env and/or Gag proteins expressing cells. 150 to 200 virus-expressing cells were counted in each case, and results are expressed as the number of syncytial cells out of 100 viral proteins expressing cells.

Results

hDlg binds to HTLV-1 envelope glycoproteins

To identify cytosolic binding partners of the HTLV-1 Env, we performed a yeast two-hybrid screen using the cytoplasmic domain of the viral protein (Env-CD) as a bait. L40 yeast cells were co-transformed by a plasmid encoding a chimera between Env-CD and the LexA transcription factor DNA binding domain, and a PBL cDNA library fused in frame to cDNA of the Gal4 activating domain. After screening, one of the isolated clones was found to correspond to a fragment of hDlg. This sequence encompassed two of the three PDZ domains and the SH3 domain of hDlg (Fig. 1A), indicating that this part of the protein was sufficient to mediate the interaction with the HTLV-1 Env cytoplasmic tail in yeast.

PDZ domains of hDlg bind to the C-terminal residues of HTLV-1 Env-CD

SH3 domains are known to interact with proline-rich sequences, whereas PDZ domains interact with the C-terminal consensus sequence -E/D-S/T-x-V/L/I. HTLV-1 Env-CD does not contain any proline-rich stretch, but its C-terminus fits with the canonical motif for interaction with PDZ domains (Fig. 1B). Hence, we tested whether the three PDZ domains alone

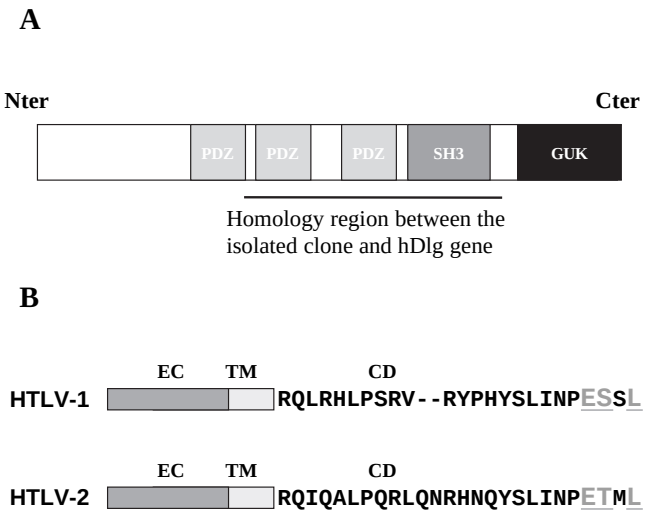


Fig. 1. Schematic representation of hDlg and HTLV-1/HTLV-2 envelope transmembrane proteins. (A) Like other members of the MAGUK subfamily, hDlg is composed of three PDZ domains, one SH3, and one Guanylate kinase-like (GUK) domain. In addition, hDlg possesses a unique proline-rich N-terminal region. The portion of hDlg isolated during the yeast two-hybrid screen is indicated. (B) The transmembrane subunits of HTLV-1 and HTLV-2 envelope glycoproteins are composed of an extracellular domain (EC), a transmembrane domain (TM), and a cytoplasmic domain (CD). The entire primary sequences of the cytoplasmic domains are shown. Conserved consensus motifs involved in the interaction with PDZ domains are shown in grey and underlined.

were sufficient to mediate binding with HTLV-1 Env-CD in the two-hybrid assay. We found that HTLV-1 Env-CD can interact with hDlg PDZ domains as well as with the full-length protein (Fig. 2A). Binding was specific because no association was detected between HTLV-1 Env-CD and the lamin protein used as an irrelevant control (Fig. 2A).

Next, we investigated whether the -E-S-S-L sequence located at the C-terminus of the HTLV-1 Env CD is required for the interaction with hDlg. We produced a HTLV-1 Env CD mutant lacking the entire PDZ-binding motif (ΔESSL) using site-directed mutagenesis and examined its capacity to bind hDlg in the two-hybrid assay. We also tested our collection of HTLV-1 Env-CD point mutants (Delamarre et al., 1999). Deletion of the entire ESSL motif or single amino acid substitution in the ESSL motif of either glutamic acid with alanine (E484A), serine with leucine (S486L), or leucine with glutamine (L488Q), were sufficient to abrogate the interaction between HTLV-1 Env-CD and hDlg (Fig. 2B). In contrast, mutation of tyrosine residues at position 476 or 479 (Y476S and Y479S mutants) had no effect (data not shown). Taken together, these results indicate that the interaction between Env-CD and hDlg was mediated through the direct binding between the PDZ domains of hDlg and the C-terminal motif of HTLV-1 envelope CD.

Interaction with hDlg is conserved in HTLV-2 envelope glycoproteins

HTLV-1 is closely related to HTLV-2 and to bovine leukemia virus (BLV), these three viruses belonging to the same genus of oncoretroviruses. However, only HTLV-2 Env-CD contains the C-terminal consensus-binding motif (Fig. 1B). We performed a yeast two-hybrid assay to determine whether the interaction between envelope CDs and hDlg was conserved within the HTLV/BLV genus. As shown in Fig. 2C, HTLV-2 Env-CD interacted with hDlg whereas BLV Env-CD did not. Murine leukemia virus (MuLV), human immunodeficiency virus type-1 (HIV-1), and simian immunodeficiency virus glycoproteins (SIV) CDs, which lack a consensus PDZ binding motif, were also unable to interact with hDlg (not shown). These results demonstrate that a specific C-terminal consensus sequence present in HTLV-1 and HTLV-2 Env-CDs is required for the interaction with hDlg. A search in databases was then carried out to identify other viral glycoproteins that possess a putative PDZ-binding C-terminal motif. Only two other viral glycoproteins, the gp340 of Epstein-Barr virus and the G protein of most rabies virus strains, were found to match with this criterion. Indeed, the CDs of both proteins interacted with hDlg in the yeast two-hybrid assay (Fig. 2D). Thus, the CDs of HTLV-1 and HTLV-2 envelope glycoproteins contain a conserved PDZ-binding domain that permits their association with hDlg in vitro. Two non-related virus families also share

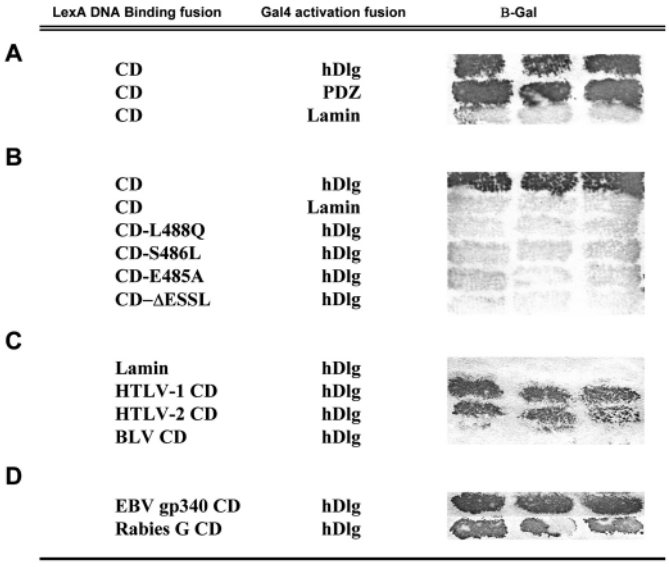


Fig. 2. Interaction between the cytoplasmic domains of several virus envelope glycoproteins and hDlg as determined by yeast two-hybrid assay. Yeast strain L40 was co-transformed with a plasmid encoding the fusion protein between the cytoplasmic domain of viral envelope and the LexA DNA-binding domain, and a plasmid encoding the fusion protein between hDlg (or its PDZ domains) and the Gal4 activation domain. Interaction between both fusion proteins leads to the activation of the *lacZ* reporter gene, which was visualized by the β-galactosidase assay. Lamin protein was used as a negative control. (A) Interactions between the cytoplasmic domain of HTLV-1 envelope glycoproteins (CD) and the full-length hDlg protein (hDlg) or a segment of hDlg protein containing three PDZ domains (PDZ). (B) Interactions between mutated CDs and hDlg. (C) Interactions between envelope CDs of various members of the HTLV/BLV retrovirus genus and hDlg. (D) Interactions between hDlg and the CDs of Epstein-Barr virus (EBV) gp340 and rabies virus G glycoprotein.

this binding property. Together, these results suggest that hDlg and/or other MAGUKs may play an essential role during the replication of various viruses in host cells.

hDlg binds to native HTLV-1 envelope transmembrane subunit

We next determined if HTLV-1 envelope CD could associate with hDlg produced in human T lymphocytes. Jurkat T cells were lysed by freeze/thaw cycles, and the resulting cytosolic extracts were incubated either with GST fused to HTLV-1 Env-CD (GST-CD) or GST alone. The associated proteins were detected by immunoblotting using an anti-hDlg mAb. Endogenous hDlg interacted with the GST-CD fusion protein but not with the GST alone (Fig. 3A). We confirmed this finding using a monoclonal antibody directed against the PDZ domains of PSD95, which recognizes several Dlg-like MAGUKs including hDlg and PSD95. The GST-CD fusion protein pulled-down a 120 kDa protein, most likely hDlg, as well as several proteins with lower molecular weight (Fig. 3B), suggesting that HTLV-1 Env directly interacts not only with hDlg but also with other MAGUKs. Alternatively, the presence of additional bands might reflect the ability of hDlg to bind other PDZ-domain containing proteins, as previously shown (Nix et al., 2000). In any event, the fact that hDlg was the major protein detected strongly suggests that hDlg is the predominant binding partner of HTLV-1 Env-CD.

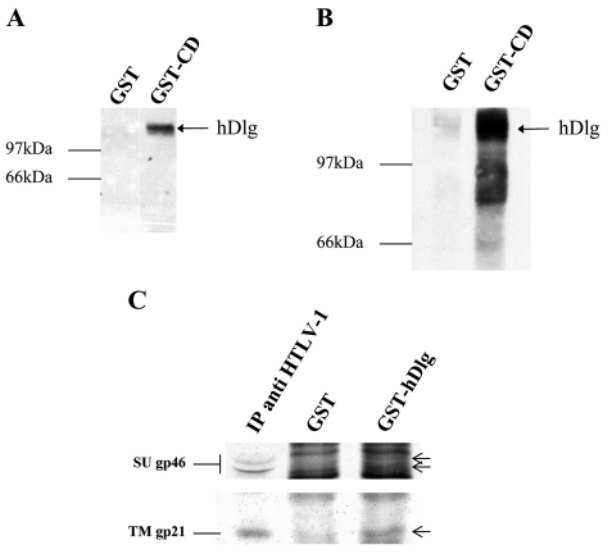


Fig. 3. Interaction between native HTLV-1 envelope proteins and hDlg. (A,B) Jurkat cells were lysed by several freeze/thaw cycles, and membrane and cytosol fractions were separated by centrifugation. The cytosol fraction was submitted to precipitation with either GST alone or GST fused to the CD of HTLV-1 envelope proteins (GST-CD). The precipitated proteins were detected either using a mAb specific for hDlg (A) or a mAb directed against the PDZ domains of MAGUKs (B). (C) Primary HTLV-1 infected CIB T-cells were metabolically labelled for 16 hours and lysed in a 0.5% NP40 buffer. Soluble material was subjected to precipitation with either GST alone or GST fused with the full-length hDlg (GST-hDlg). As a control, viral proteins were immunoprecipitated from cell lysate using sera obtained from HTLV-1 infected patients (IP anti-HTLV-1).

We then performed reciprocal experiments to investigate whether hDlg could associate with the HTLV-1 Env-CD in the context of native envelope proteins. Using GST-hDlg beads, we precipitated proteins from lysates of short-term cultured HTLV-1-infected T cells (CIB cells). Under these conditions, the TM subunit of HTLV-1 envelope glycoproteins (gp21) associated with GST-hDlg (Fig. 3C). This interaction was specific because the TM did not associate with the GST alone (Fig. 3C, compare GST with GST-hDlg). We also detected a fraction of the SU subunit (Fig. 3C, compare GST and GST-hDlg) that might correspond to SU co-precipitated with the GST-hDlg/TM complexes via heterodimerization with the TM. This strongly suggests that it was indeed the SU/TM envelope complex in its native conformation that bound hDlg. Hence, endogenous hDlg and native HTLV-1 Env-CD associate with their GST fusion counterparts, thus confirming the yeast two-hybrid analysis.

HTLV-1 Env and hDlg co-localized in restricted areas of the plasma membrane of HTLV-1-infected T lymphocytes

By using the two hybrid and pull down assays, we suggested that HTLV-1 Env and hDlg were binding partners. To investigate whether this association also occurs in vivo, we next performed confocal immunofluorescence localization studies in HTLV-1-infected primary CIB T cells. At steady state, HTLV-1 envelope glycoproteins appeared as clusters at the plasma membrane (Fig. 4A, Env). hDlg was enriched in HTLV-1 Env-positive areas of the plasma membrane, although a significant amount of hDlg was also distributed diffusely throughout the cytoplasm (Fig. 4A, hDlg). That a fraction of hDlg co-localizes with Env at the plasma membrane of infected T lymphocytes provides another strong argument that the two proteins are associated. Moreover, it confirms that hDlg interacts with native SU/TM heterodimers (Fig. 3C), because mature SU/TM heterodimers are the only envelope products detected at the plasma membrane of infected cells (Pique et al., 1992; Pique et al., 1993).

Beside hDlg, we also found that the Gag precursor proteins responsible for virus assembly and budding were clustered in Env positive areas (Fig. 4B). This strongly suggests that the restricted sites of the plasma membrane of infected T-lymphocytes that are enriched in Env, Gag and hDlg serve as platforms for virus assembly and release.

Membrane clusters containing HTLV-1 Env glycoproteins have characteristics of T-cell-contact sites

The distribution of hDlg we found in HTLV-1-infected primary T cells contrasted with that found in uninfected resting T cells where hDlg exhibits a diffuse distribution in the cytoplasm, and was rather reminiscent of the pattern found in T cells upon cross-linking of CD2 that mimics cell-cell contacts (Hanada et al., 2000). As all HTLV-1-infected cells, CIB cells express higher levels of surface adhesion molecules than non-infected T cells (Kambara et al., 1999; Tanaka, 1999; Valentin et al., 1997) and as a result grow as tight clusters in culture. Thus, we postulated that plasma membrane areas where Env and hDlg co-localize correspond to former cell-cell contact sites mechanically disrupted before cell transfer onto glass slides.

To test this hypothesis, we examined whether the HTLV-1 Env-positive areas also contain proteins known to be present in specialized T-cell contact sites, due to their functions in either antigen recognition (CD4), cell adhesion (CD2) or cell activation and signal transduction (CD25 and $p56^{lck}$) (Bromley et al., 2001; Revy et al., 2001). Because T-cell contact sites contain lipid rafts, cells were also labelled with Cholera toxin that specifically binds the raft marker GM1 (Parton, 1994).

We found that HTLV-1 Env-positive areas at the plasma membrane of HTLV-1-infected primary T lymphocytes contained CD2 and $p56^{lck}$ (Fig. 4C, CD2 and Lck panels), in agreement with previous studies (Hanada et al., 1997; Hanada et al., 2000). Env-positive structures were also enriched in both CD25 and CD4 (Fig. 4C, CD25 panel and data not shown) (Krummel and Davis, 2002). Finally, we also found enrichment in lipid rafts, as evidenced by the high degree of co-localization between Env and Cholera toxin (Fig. 4C, GM1 panel), consistent with previous reports linking retrovirus assembly and lipid rafts (Feng et al., 2003; Ono and Freed, 2001). Taken together, these results indicate that HTLV-1 Env and hDlg are

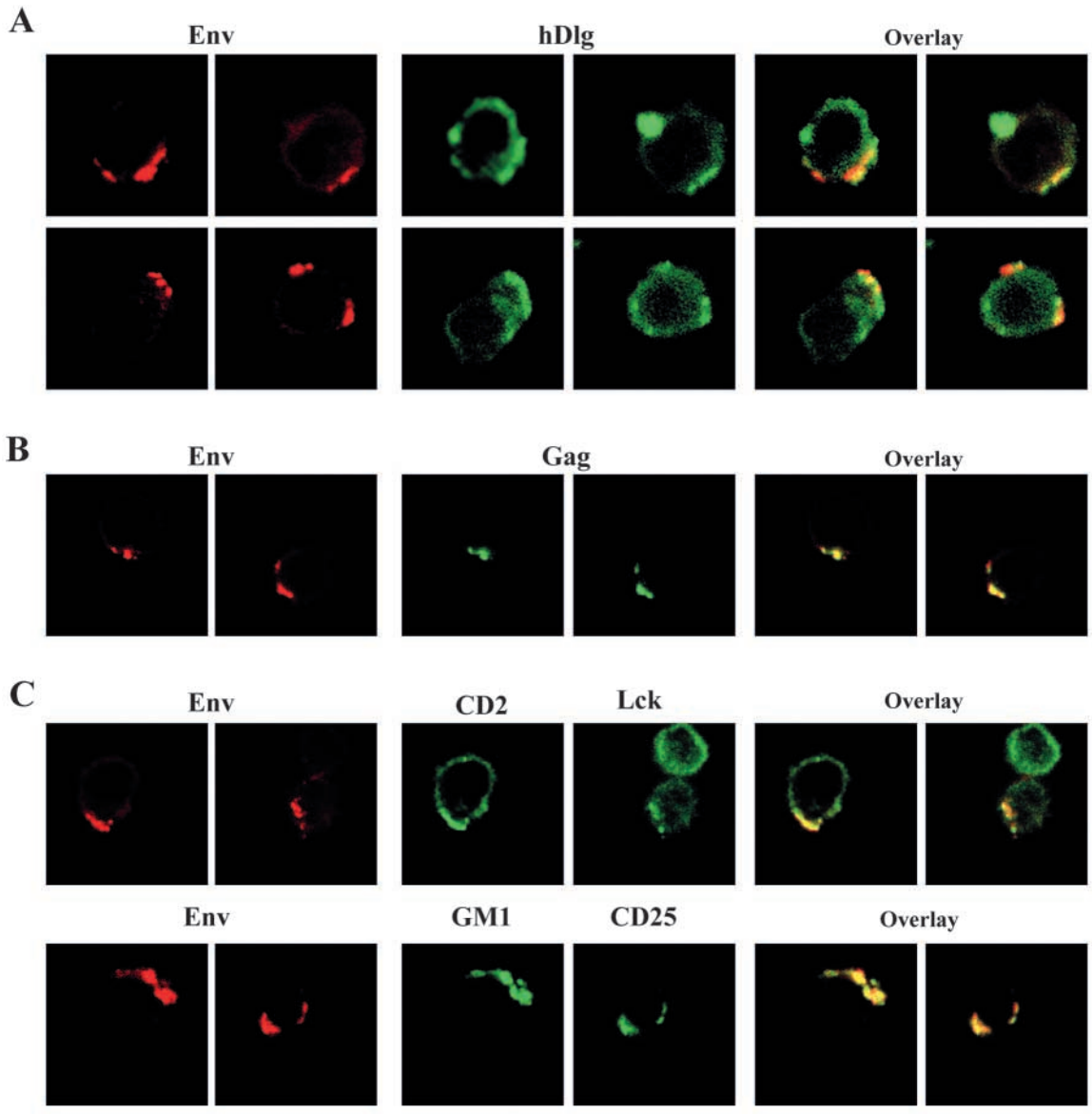


Fig. 4. Concentration of HTLV-1 envelope glycoproteins and hDlg in restricted areas characteristic of cell contact sites in HTLV-1-infected primary T lymphocytes. Each row shows a single optical plane of HTLV-1 infected CIB cells, as recorded independently in the red and green channels by confocal microscopy. (A,B) HTLV-1 envelope glycoproteins were stained using sera obtained from infected patients, and Texas-red conjugated secondary antibodies before permeabilization (left panels). hDlg (A) and Gag (B) were stained on saponin-permeabilized cells using 2D11 and p19 monoclonal antibodies, respectively, followed by FITC-conjugated secondary antibodies (middle panels). On the right panels are presented overlay profiles of red and green channels (Overlay panels). Co-localization signal appears as yellow pixels. (B) HTLV-1 envelope glycoproteins were stained as described above (left panels). Staining of CD25, CD2 and CD4 were performed on non-permeabilized cells using either FITC-conjugated mAb, or mAb followed by FITC-conjugated secondary antibodies (middle panels). Staining of GM1 was done on non-permeabilized cells with biotin-conjugated cholera toxin and DTAF-conjugated streptavidin. Staining of Lck was performed on saponin-permeabilized cells using rabbit sera followed by FITC-conjugated secondary antibodies. Overlay profiles of red and green channels. Co-localization signal appears as yellow pixels (right panels, merge).

present into polarized structures at the plasma membrane of infected primary T cells that have characteristics of T-cell contact areas and serve as virus assembly sites.

Mutant HTLV-1 unable to interact with hDlg display a reduced ability to trigger cell-to-cell fusion between T lymphocytes

We finally sought to determine the functional role of Env/hDlg interaction in HTLV-1 life cycle. As hDlg and Env appear to be concentrated in cell contact areas, we compared the characteristics of T-cell contacts formed between target cells and cells expressing either wild-type or mutant HTLV-1 in which Env proteins are unable to interact with hDlg.

Jurkat T cells were thus transfected with either the wild-type provirus (XMT-WT) or mutated proviral constructs, in which the ESSL carboxy-terminus PDZ binding motif in Env was disrupted (XMT-Env-S486L, Fig. 2B), or in which a premature stop codon was introduced in the *env* gene resulting in an Env deleted virus (XMT-delta Env) (Delamarre et al., 1999). Detection of viral Env and/or Gag proteins by immunofluorescence indicated that in each case, approximately 10% to 15% of the cells expressed viral proteins (data not shown). Tight cell clusters were present in cultures of cells transfected with either the WT or the Env-S468L XMT provirus (Fig. 5A, XMT-WT and XMT-Env-S486L) but absent in cultures of cells transfected with the Env deleted virus (Fig. 5A, XMT-delta Env). This indicates that expression of Env permitted the establishment of cell-cell contacts, probably due to Env/receptor(s) interaction.

In cultures of cells expressing the XMT-WT virus, we easily detected gigantic cells associated with clusters (Fig. 5A, arrowheads) that are most probably syncytial cells resulting from cell-cell fusion events. However, much less of these gigantic cells were found in culture of cells expressing the XMT-Env-S486L provirus (Fig. 5A). This strongly suggested that, although dispensable for early cell conjugates formation, Env/hDlg interaction might be essential for subsequent Env-mediated cell fusion. To test this hypothesis quantitatively, we concentrated transfected T cells to facilitate the formation of stable conjugates, as recently described (Igakura et al., 2003). We then compared Env and Gag localizations and evaluated the number of syncytia in cultures of cells transfected with WT, Env-S486L or delta-Env HTLV-1 provirus.

In preserved cell clusters, Gag and Env proteins were found concentrated at the sites of T-cell contacts, strongly suggesting that typical virological synapses have been formed (Fig. 5B, XMT-WT). Importantly, whether envelope glycoproteins were expressed or not, Gag appeared concentrated in T-cell contact sites (Fig. 5B, Gag panels, XMT-WT, XMT-Env-S486L, XMT-delta Env). This indicates that Env did not contribute to Gag concentration at the virological synapse. In addition, Gag co-localized extensively with Env proteins at the cell surface, whether envelope proteins conserved their PDZ-binding motif or not (Fig. 5B, Env and Gag panels, XMT-WT and XMT-Env-S486L), thus excluding a potential role of hDlg in Env/Gag clustering.

We then estimated the abilities of wild-type and mutated viruses to trigger syncytia formation between T cells. To do so, we counted cells containing more than one nucleus among all the viral Env and/or Gag proteins expressing cells. Although the cell surface expression level of S486L mutated envelope glycoproteins was comparable to that of the wild-type Env (Fig. 5B, Env panels), we noticed a strong decreased in the ability of the S486L mutated virus to induce syncytia formation as compared to the wild type. Indeed, only 12% and 15% of S486L virus expressing cells were syncytia after 30 minutes and 90 minutes of cell contacts, respectively, as compared with 34% and 35% in culture of cells expressing the wild-type virus. Moreover, while most syncytia induced after expression of the wild type HTLV-1 virus contained at least 4 nuclei (Fig. 5B, XMT-WT), syncytia induced by the S486L virus contained a maximum of 2 nuclei (XMT-Env-S486L, lower panel), suggesting that they result from more rare fusion events. No syncytia were detectable in cells expressing the delta-Env provirus (Fig. 5B, XMT-delta-Env), indicating that the syncytia we described above were indeed dependent of HTLV-1-Env functions. Hence, preventing Env/hDlg interaction in the context of the full-length provirus expressed in T lymphocytes decreases the ability of Env to trigger cell-to-cell fusion.

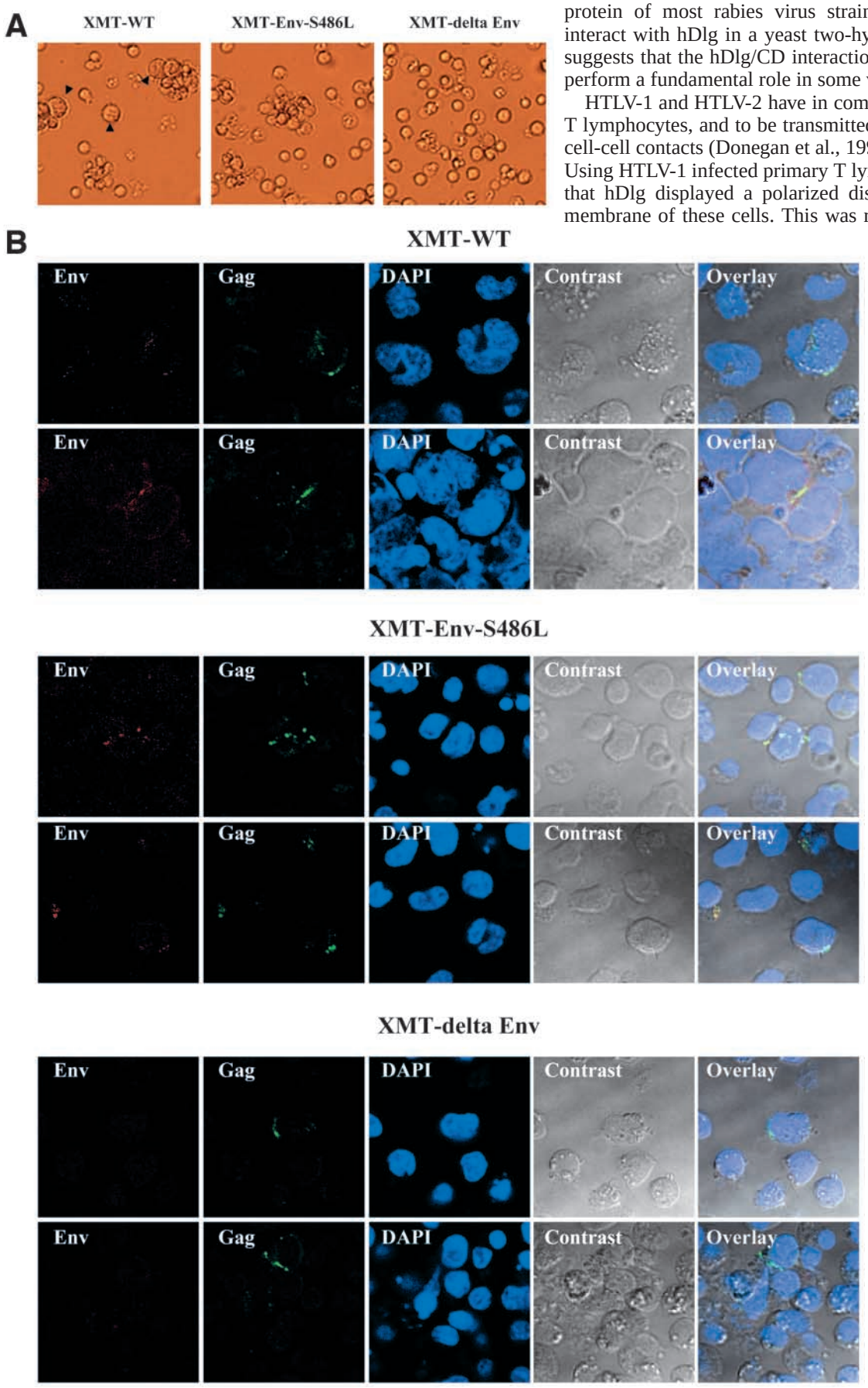
Discussion

Here, we report a novel interaction between the scaffolding protein hDlg and the envelope glycoproteins of HTLV-1. We demonstrate that hDlg binds to the cytoplasmic domain of the HTLV-1 Env and that the two proteins are concentrated in cell contact sites at the plasma membrane of infected T-lymphocytes. We also show that preventing Env/hDlg interaction in the context of a complete HTLV-1 virus leads to decreased ability of Env to trigger cell-to-cell fusion between T lymphocytes. These findings constitute the first example of a functional interaction between a MAGUK family member and a viral structural protein.

Like other members of this subfamily of MAGUKs, hDlg is composed of protein interaction modules including three PDZ domains, a central SH3 domain, and a C-terminal GUK domain (Gonzalez-Mariscal et al., 2000). Sequence analysis showed that the four C-terminal residues of Env-CD (E-S-S-L) were conserved between the most distant HTLV-1 strains (Gessain et al., 1993; Malik et al., 1988), and fit with the consensus sequence (E/D-S/T-x-V/L/I) required for binding to the PDZ domains. Using a yeast two-hybrid assay, we found that the Env/hDlg interaction was indeed mediated through the canonical PDZ-interacting motif of Env and the PDZ domains of hDlg. Hence, it appears that the virus has evolved to mimic the interaction between hDlg and cell surface receptors.

In addition to HTLV-1, we found that HTLV-2 Env glycoproteins have the ability to interact with hDlg in the yeast two-hybrid assay. This interaction also occurred via a canonical motif for interaction with PDZ domains present at the C-terminus of HTLV-2 Env-CD. HTLV-2 is a virus of the same genus as HTLV-1 and both viruses share 60% homology at the amino acid level. The fact that HTLV-1 and HTLV-2 envelope glycoproteins conserved the ability to interact with hDlg suggests that this interaction plays an important role in the life cycles of these retroviruses. However, Env-hDlg interaction is not a general paradigm for all retroviruses, because the cytoplasmic domains of BLV, HIV, SIV and MuLV envelope glycoproteins failed to bind to hDlg in yeast two-hybrid assays.

Besides HTLV-1 and HTLV-2 Env, only two other viral glycoproteins, the gp340 of Epstein-Barr virus and the G



protein of most rabies virus strains, were found able to interact with hDlg in a yeast two-hybrid assay. This finding suggests that the hDlg/CD interaction could have evolved to perform a fundamental role in some viruses but not in others.

HTLV-1 and HTLV-2 have in common the ability to infect T lymphocytes, and to be transmitted in vivo exclusively via cell-cell contacts (Donegan et al., 1994; Igakura et al., 2003). Using HTLV-1 infected primary T lymphocytes, we observed that hDlg displayed a polarized distribution at the plasma membrane of these cells. This was reminiscent of what was

Fig. 5. Mutant HTLV-1 unable to interact with hDlg shows reduced Env-triggered syncytia formation between T-lymphocytes. (A) Images of Jurkat T cells in culture 36 h after transfection with either wild-type (XMT-WT), envelope mutated (XMT-Env-S486L) or envelope deleted (XMT-delta Env) HTLV-1 provirus. The arrowheads point to gigantic syncitia detectable in cultures of cells expressing wild-type HTLV-1. (B) Thirty six hours after transfection, Jurkat T-cells were concentrated to 10^7 cells/ml and seeded onto glass slides for 30 minutes minutes to favour cell-to-cell contacts between transfected and nontransfected cells. Viral Env and Gag proteins are detected as described in the legend of Fig. 4A. The left rows show single optical planes as recorded in the red (Env) and green (Gag) channels by confocal microscopy; DAPI staining showing cells nucleus (DAPI) and a contrast phase recording showing the overall cell structure (contrast) are also presented.

previously observed upon CD2 cross-linking in normal lymphocytes, which provokes the translocation of a significant amount of hDlg from the cytosol to the caps formed at the cell contact sites (Hanada et al., 2000). Owing to over-expression of several adhesion molecules (Kambara et al., 1999; Tanaka, 1999; Valentin et al., 1997), HTLV-1 chronically infected cells grow as tight clusters in culture, creating constant cell contacts. In vivo, HTLV-1 transmission through cell-cell contacts could occur during antigen recognition, as illustrated by the preferential infection of T cells directed to HTLV-1 epitopes (Goon et al., 2004; Hanon et al., 2000). Hence, hDlg may be constantly recruited to the plasma membrane due to frequent cell-cell contacts encountered in HTLV-1-infected cells both in vivo and in vitro culture conditions.

In HTLV-1-infected primary T cells, we found that the distribution of HTLV-1 Env proteins was also polarized, and that Env partially co-localized with hDlg. The Env-positive membrane clusters also contained p56lck and CD2, whose interaction and co-localization with hDlg has been previously reported (Hanada et al., 1997; Hanada et al., 2000). Furthermore, we found that CD4, CD25 and lipid rafts were also present in these membrane clusters, a combination of characteristics reminiscent of the immunological synapse, the region of contact between T lymphocytes and antigen presenting cells (Bromley et al., 2001). These results are therefore consistent with the previous suggestion that hDlg could be recruited to the immunological synapse (Hanada et al., 2000).

The Gag precursor protein is the only viral component responsible for virus assembly and budding. As evidenced here, Gag proteins were also found polarized at the plasma of infected T lymphocytes where they co-localized with Env. Hence, the plasma membrane areas of infected T lymphocytes where hDlg and Env co-localized displayed the expected characteristics of HTLV-1 assembly platforms. These membrane areas were found highly enriched in lipid rafts, in agreement to previous reports demonstrating the role of these microdomains in the assembly process of different retroviruses including HTLV-1 (Feng et al., 2003; Ono and Freed, 2001). Moreover, observation of cell clusters between HTLV-1 productive and target Jurkat cells showed that viral assembly platforms were concentrated precisely at the sites of cell-cell contacts. These structures were recently demonstrated to represent the sites where HTLV-1 cell-to-cell transmission occurs and were called ‘virological synapses’ (Igakura et al., 2003). We conclude therefore that similarly to its functional implication in the neurological and perhaps immunological synapses (Fujita and Kurachi, 2000; Hanada et al., 2000), hDlg could also be a member of the HTLV-1 virological synapse.

hDlg functions by clustering several integral membrane proteins into cell contact sites, preventing their diffusion within the plasma membrane, hence participating to the organization and stabilization of cell-cell contacts areas (Fujita and Kurachi, 2000). We thus tested the importance of hDlg/Env interaction in the establishment and stability of the HTLV-1 virological synapse.

We first found that Gag proteins were concentrated to sites of cell-cell contacts, whether the envelope glycoproteins were present or absent. This reveals that Env proteins do not directly participate to the early cell-cell contact processes that lead to the establishment of the virological synapse. Moreover, regardless of the conservation of their PDZ-binding motif, envelope glycoproteins co-localized with Gag at the cell surface, indicating that hDlg is not involved in the intracellular trafficking of Env towards the site of virus assembly.

However, Env/hDlg interaction was found to profoundly influence the ability of HTLV-1-producing T lymphocytes to undergo cell-cell fusion. Indeed, T cells expressing a mutated HTLV-1 with envelope proteins unable to bind hDlg displayed a strongly reduced ability to induce syncytia formation with target cells, as compared with those expressing a wild-type virus and despite comparable Env cell surface expression levels. Interestingly, we had previously shown that the ESSL PDZ-binding motif play no role in Env ability to induce syncytia formation between adherent cell lines (Delamarre et al., 1999). This first indicates that the mutation of the ESSL PDZ binding motif in Env had no intrinsic effects on the global fusiogenic ability of Env. This also suggests that although hDlg is dispensable for Env mediated fusion when cells are artificially maintained in contacts, like adherent cells growing side by side in a plastic dish, Env/hDlg interaction becomes essential when cell conjugates must be formed naturally, like between T lymphocytes. Importantly, HTLV-1 is mainly transmitted through cell contacts involving T lymphocytes in vivo. We therefore propose that, through the stabilization of envelope complexes at the virological synapse, hDlg could increase the stability of cell conjugates between HTLV-1 infected and target cells which in turn would optimize Env mediated membrane fusion and subsequent viral spread.

In conclusion, we have shown here that a member of the MAGUK family interacts with the envelope glycoproteins of HTLV-1 and that this interaction is essential for efficient T-lymphocyte cell fusion, an event correlated the cell-to-cell transmission of this virus in vivo. Interestingly, rabies viruses are transmitted from neuron to neuron in a transynaptic manner, and their G proteins, which were also shown here to bind hDlg, are absolutely required in this process (Etessami et al., 2000). This opens the exciting possibility that MAGUK proteins might represent essential cellular factors that assist the peculiar cell-to-cell transmission process of several unrelated viruses.

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